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Adrenogenital Syndrome Due to Enzymatic Defects in Cortisol Synthesis

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CONGENITAL virilizing adrenal hyperplasia is a fascinating disorder, partly because it causes spectacular alterations in sexual development which now can be prevented or dramatically reversed by treatment with cortisone and partly because it is an "experiment of nature," not producible in the laboratory, which helps us to understand the normal biosynthesis and physiology of adrenocortical hormones. It is also one of the best understood of the genetically determined enzymatic defects.

Clinical studies have shown that there are three different types of virilizing adrenal hyperplasia causing (1) simple virilism, (2) virilism with hypertension and (3) virilism with tendency to sodium-loss. Their relative frequency in our clinic is shown in Table I. There is a high familial incidence of the disorder. Each of these types may be due to a different specific defect of enzymes concerned in the biosynthesis of adrenal hormones, since in each family in which the disorder occurs all the children who are affected have the same type of disease. The table suggests, at first glance, that three or four times more females than males are affected. Childs,[1] however, believes that this impression may be biased by the fact that the disease is more apt to pass undiagnosed in the male. Eliminating the so-called "index cases" which present in the clinic, he found that the affected sibs of these patients have an approximately equal sex distribution as shown in Table II.

Bartter and his associates[2] were the first to suggest that in the adrenogenital syndrome the ability of the adrenals to synthesize hydrocortisone might be impaired; Jailer[3] confirmed this by showing that ACTH caused little increase in its output. We[4] illustrated this concept with a diagram indicating that there was a partial defect in the production of glucogenic and possibly sodium-retaining hormones with the formation of abnormal amounts of

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TABLE 1
Relative Frequency and Sex Distribution of Different Types of Virilizing Adrenal Hyperplasia in Patients Studied at the Harriet Lane Home of the Johns Hopkins Hospital

Patients	Virilism Alone	Sodium-Losing	Hypertensive	Total
Females...	54*	32	5	91
Males.....	12	8	2	22

* 9 raised as males.

TABLE II
Ratio of Male to Female Patients with Virilizing Adrenal Hyperplasia—Children's Medical Center, Boston. From: CHILDS, B. *Pediatrics*, 25: 565, 1960.

Patients	Males	Females	Total	Sex Ratio M:F
Index cases.	30	84	114	0.36
Affected sibs.....	14	18	32	0.78
Total.....	44	102	146	0.43

TABLE III
Methods of Studying the Adrenogenital Syndrome

1. Effect of ACTH stimulation on { plasma steroids / urinary metabolites }
2. Suppression of adrenals by cortisone and analogues.
3. Administration of precursors to patients.
4. Tissue slice synthesis—C^{14} precursors.
5. SU-4885 (C-11-hydroxylation) block.
6. Aldosterone measurements—under sodium deprivation.

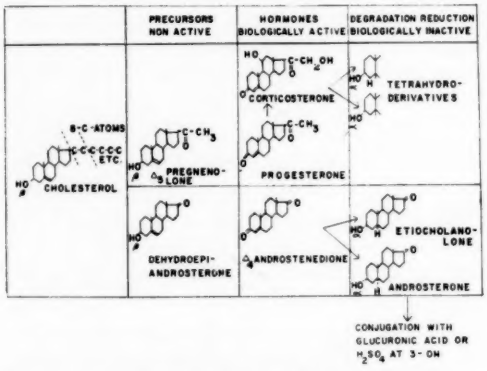

FIG. 1. Chemical structure of active steroidal hormones, their precursors and their degradation products.

androgenic and estrogenic steroids. To compensate for the relative deficiency of hydrocortisone, the activity of the adrenal was whipped up by increased secretion of pituitary ACTH. The latter was demonstrated by Sydnor and his colleagues.[5] It remained, however, to determine more specifically the actual abnormalities of steroidogenesis.

METHODS OF STUDY

The methods which have been applied to the study of virilizing adrenal hyperplasia are listed in Table III. It was recognized early that the concentration of compound F (cortisol) in the plasma and the output of its metabolites tetrahydro-hydrocortisone (THF) and tetrahydrocortisone (THE) in the urine were decreased and that the administration of ACTH did not increase these products to the extent that it does in normal persons. This might be due, of course, to the fact that the adrenals are already working under maximal ACTH stimulation. However, the urine was shown to contain relatively large amounts of steroids not found in significant quantities in normal urine and the amounts of these abnormal steroids were increased by the administration of ACTH indicating that the adrenals were responsive.[6] The question was raised of whether these abnormal metabolites were due to an abnormality in the hepatic degradation of adrenal steroids or whether they are expected degradation products of certain precursors of hydrocortisone which accumulate in excess because of a block in its synthesis. Recent work[7] indicates that, although in the adrenogenital syndrome there may be some minor alterations in hepatic degradation, these are of relatively little importance compared to the block of synthesis of cortisol. The disappearance of the abnormal steroids from the urine when adrenal activity is suppressed by the administration of physiologic amounts of cortisone or its analogues lends further support to this hypothesis.

I shall mention also some interesting studies which have been carried out by administering the chemical SU-4885 which blocks C_{11}-hydroxylation to patients with virilizing adrenal hyperplasia and some studies of aldosterone excretions in patients with the salt-losing type.

PATHWAYS OF STEROIDAL SYNTHESIS AND DEGRADATION

Figure 1 presents the general scheme for the formation in the adrenals or gonads of biologically active steroids from their inactive pre-

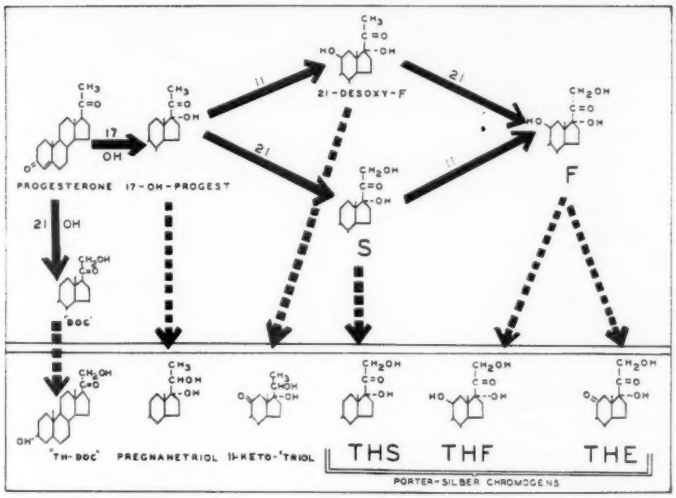

FIG. 2. Pathway of biosynthesis of hydrocortisone showing intermediary precursors and the 21-carbon urinary metabolites derived from them.

cursors followed by their subsequent degradation in the liver to inactive metabolites which are conjugated to soluble compounds readily excreted in the urine. Cholesterol is first converted into 21-carbon or 19-carbon steroids by the removal of six or eight of the carbon atoms attached at C_{17}. Δ_5-pregnenolone and dehydroepiandrosterone are both inactive steroids having, like cholesterol, a double bond at $C_{5,6}$ and a β—OH at C_3. For the formation of biologically active steroids, such as progesterone and Δ_4-androstene-dione, the double bond must be shifted to C_{4-5} and the hydroxyl at C_3 converted to ketone. The degradation of these hormones to inactive metabolites consists of reduction of the A ring with removal of the double bond and the conversion of the C_3 ketone to a 3-α-hydroxyl, which is afterward conjugated with glucuronic or sulfuric acid. Theoretically, a defect of steroidogenesis could be due to a defect of conversion of ΔC_5 to ΔC_4 (since this paper was presented a case of congenital adrenal hyperplasia with this defect has been published by Bongiovanni[19]).

Figure 2 is a simplified scheme showing the synthesis of hydrocortisone from progesterone and the urinary 21-carbon metabolite derived from each of the intermediary precursors. The solid arrows indicate the hydroxylations which occur at the 17-, 11- and 21-carbon atoms. The dotted arrows indicate the corresponding end metabolite derived from each of the precursors of compound F (hydrocortisone). Some of the intermediary compounds in the formation of these metabolites have purposely been omitted. From this chart, one can predict which metabolites one would expect to find when there is a defect of C_{21}-hydroxylation, C_{11}-hydroxylation or both of these hydroxylations.

Less is known about the synthesis of the 19-carbon steroids, or androgens, of the adrenal or the derivation of the 19-carbon metabolites, the 17-ketosteroids. Figure 3 shows (lower section) the probable synthesis of Δ_4-androstenedione and 11-keto-androstenedione from dehydroepiandrosterone. The 19-carbon urinary metabolites (17-ketosteroids) which are principally androsterone and etiocholanolone and their 11-oxy derivations, are shown in the middle section. As indicated by the dotted arrows, a portion of these metabolites can be derived from the 21-carbon steroids (upper line) as well as from the 19-carbon steroids (lower line). However, the 21-carbon steroids make only a minor contribution, probably not over 5 to 10 per cent to the total 19-carbon metabolites.[7]

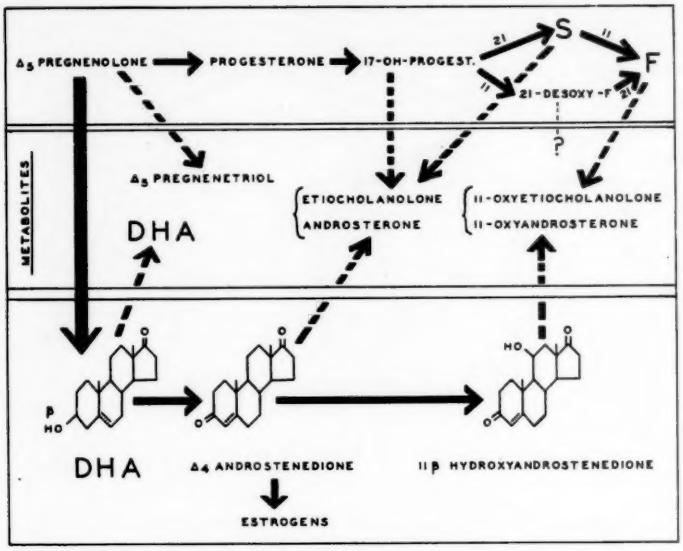

FIG. 3. Urinary 19-carbon metabolites showing their derivation from both 19-carbon and 21-carbon steroids.

THE C-21-HYDROXYLATION DEFECT IN VIRILIZING ADRENAL HYPERPLASIA

The simple virilizing form of adrenal hyperplasia is due primarily to a defect in 21-hydroxylation as shown in Figure 4. With this defect the precursors of cortisol, 17-OH-progesterone and 21-desoxy-F are increased, giving rise to increased urinary output of both pregnanetriol and the 11-oxypregnanetriols.

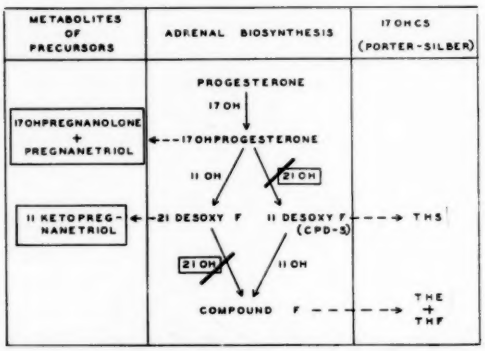

FIG. 4. Steroid synthesis in nonhypertensive adrenal hyperplasia. 21-Hydroxylase defect in simple virilizing and sodium-losing types of congenital adrenal hyperplasia. Note that 11-OH-pregnanolone, pregnanentriol and 11-ketopregnanetriol are increased while THE, THF and THS are decreased.

There is also increased excretion of the 17-ketosteroids and 11-oxy-17-ketosteroids, while the 17-OH-corticosteroids (THE, THF and THS) are decreased.

THE C-11-HYDROXYLATION DEFECT IN THE HYPERTENSIVE FORM OF ADRENAL HYPERPLASIA

Eberlein and Bongiovanni[8] showed that in patients with the hypertensive form of virilizing adrenal hyperplasia, the defect is one of 11-hydroxylation rather than 21-hydroxylation.

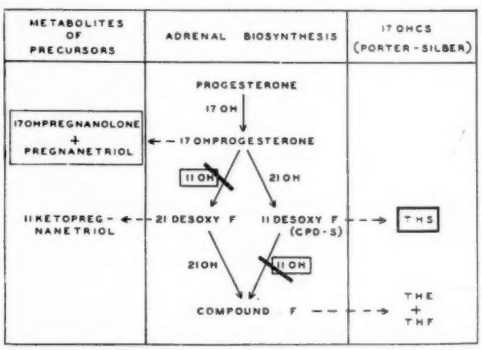

FIG. 5. 11-Hydroxylase defect in hypertensive type of virilizing adrenal hyperplasia. Note that 17-OH-pregnanolone, pregnanetriol and THS are increased while 11-ketopregnanetriol, THE and THF are decreased.

As shown in Figure 5, this gives rise to an entirely different pattern of urinary metabolites. Compound S is formed and tetrahydro-S (THS) is excreted in large amounts. Pregnanetriol is formed but 11-oxygenated pregnanetriol is missing. The 11-oxygenated 17-ketosteroids are also absent. Likewise, desoxycorticosterone (DOC) and tetrahydro-DOC are formed. These are not shown on this chart but are derived from progesterone before hydroxylation at C_{17}. The former is believed to be the cause of hypertension which disappears when the adrenal is suppressed with cortisone. However, a few patients with virilizing adrenal hyperplasia caused by the 11-hydroxylation defect have not had hypertension.

These enzyme defects are probably never entirely complete and vary somewhat in degree from one patient to another. This is illustrated by the urinary steroidal patterns of some patients studied in our laboratory by Dr. Orville Green[9] (Fig. 6 and 7). Although there are minor differences between the patients of each group, it is obvious that there is a very striking difference between the patients who are hypertensive and those who are not.

THE SODIUM-LOSING TYPE OF ADRENAL HYPERPLASIA

There is not sufficient time to discuss the still unsolved problem of the salt loss which occurs in about one-third of the patients with virilizing adrenal hyperplasia. As shown in the previous figure these patients have the C_{21} rather than the C_{11} hydroxylation defect. Bongiovanni and Eberlein[10,11] have shown that in patients with the salt-losing type of adrenal hyperplasia impairment in the formation of hydrocortisone is greater than in those with the simple virilizing type; our observations agree with this. They suggest that the difference between the two types is a quantitative rather than a qualitative one and that hydrocortisone may be necessary or "permissive" for aldosterone to exert its salt-retaining activity. On the other hand it is obvious that a complete absence of 21-hydroxylation would prevent the formation of aldosterone.

Studies of actual aldosterone excretion have been made in only a few laboratories[12–14] and there is a discrepancy in the findings. Studies of our patients[13] made during five-day periods of sodium deprivation failed to show appreciable amounts of urinary aldosterone in the patients with the salt-losing type, whereas there were possibly increased amounts in patients with the simple virilizing type. It is our opinion that possibly in patients with both the simple virilizing and the salt-losing types the ability to reabsorb sodium may be impaired and that the former compensate for this with overproduction of aldosterone while the latter cannot.

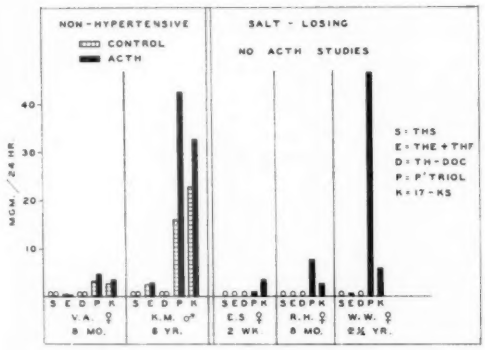

FIG. 6. Variations of urinary steroidal patterns in individual cases of the hypertensive type of virilizing adrenal hyperplasia. From: GREEN, O. C., MIGEON, C. J., and WILKINS, L. *J. Clin. Endocrinol.*, 20: 929, 1960.

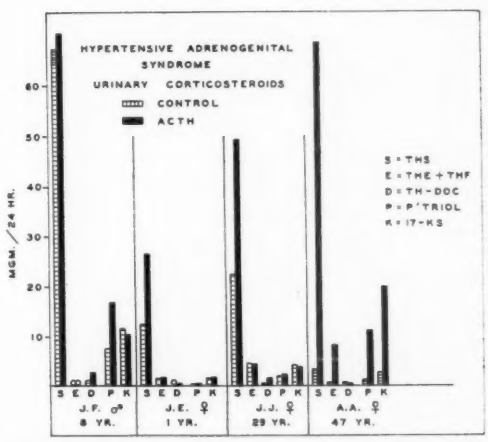

FIG. 7. Variations of urinary steroidal patterns in cases of simple virilizing and sodium-losing adrenal hyperplasia. From: GREEN, O. C., MIGEON, C. J. and WILKINS, L. *J. Clin. Endocrinol.*, 20: 929, 1960.

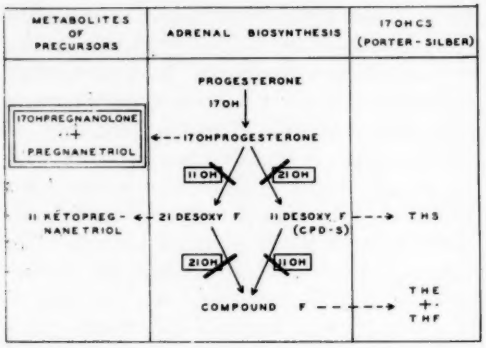

FIG. 8. Effect of SU-4885 block in patients with virlizing adrenal hyperplasia. Note that 17-OH-pregnanolone and pregnanetriol are greatly increased while 11-keto-pregnanetriol, THS, THE and THF are decreased. From: CLEVELAND, W. W. and MIGEON, C. J. *Am. J. Dis. Child.*, 100: 473, 1960.[18]

GENETIC STUDIES

In 1956, Childs and his co-workers[15] reported genetic studies made on our patients. At that time there were seventy-six subjects among 181 sibs in fifty-six families. There were no affected parents, grandparents, uncles, aunts or cousins. There was only one family in which there was consanguinity, the parents being first cousins. As stated previously, Childs[1,15] thought there was no actual difference in the sex ratio of affected patients despite the apparent marked preponderance of females. The apparent difference is probably due to the greater difficulty in recognizing the disorder in males. There was no evidence of deleterious effects on gestation or labor in the mothers nor any evidence of maternal influence on the disorder. He came to the conclusion that the disorder is due to an autosomal recessive gene which manifests itself only in the homozygote.

It would be most valuable to know the frequency of the gene in the population and to be able to detect the heterozygotous carriers of the gene. Childs and his associates,[15] making the unwarranted assumption that all patients in Maryland with the disorder born after 1939 came to our own clinic, estimated the disease occurred once per 67,000 births and calculated that the incidence of heterozygotes was one per 128 individuals of the general population. We suspect now that the incidence of the trait may be 1:50 and 1:100. Prader of Zurich[16] estimates that in Switzerland there is one patient with the adrenogenital syndrome among every 5,000 births, giving an incidence of the gene of 1:37.5 in the general population.

Prior to the treatment of adrenal hyperplasia with cortisone, the patients rarely, if ever, were fertile; with treatment, ovulation or spermatogenesis occurs. Having observed the high incidence of affected sibs in certain families, we were inclined at first to advise our treated patients not to produce children. If the genetic explanation of the disorder is correct, however, the treated patients have only a 1:100 to 1:200 chance of producing affected offspring. If a marriage with a heterozygotous carrier could be avoided, there would be no risk at all.

If the fathers and mothers of the affected subjects are heterozygotous carriers they might be expected to have a minor enzymatic defect which might be demonstrable. Since the defect in the homozygotous patients is well demonstrated by the degree to which urinary pregnanetriol increases after administration of ACTH, Childs, Grumbach and Van Wyk[15] applied this test to the study of ten mothers and ten fathers of our patients. With the exception of one father and one pregnant mother, the increment of pregnanetriol ranged from 0.4 to 3.7 mg. per day, compared with 0.0 to 4.4 mg. per day. The difference of the means was barely significant. It is of interest that in one father the pregnanetriol rose 41.3 mg.

Recently, Jailer[17] and later Cleveland et al.[18] studied the effects of introducing an 11-hydroxylation block with the chemical SU-4885 in patients with simple virilizing adrenal hyperplasia who have the 21-hydroxylation defect. This causes the output of pregnanetriol and its precursor, 17-OH-pregnanolone, to rise as high as 100 to 260 mg. per day compared to the usual output of 40 to 80 mg. per day after stimulation with ACTH. As shown in Figure 8 this is due to the fact that with the SU-4885 block the 11-keto-pregnanetriol (not measured by the usual methods) cannot be formed and all of the precursors appear as

TABLE IV

Ratios of *pregnanes*:17-OHCS in Patients with Virilizing Adrenal Hyperplasia, in Their Parents and in Normal Subjects

Subjects	Average Ratio	Range
5 patients	$\frac{145}{10.4} = 14$	(5.9–26)
6 normal subjects	$\frac{2.6}{21.1} = 0.13$	(0.10–0.18)
9 parents	$\frac{3.3}{28.0} = 0.13$	(0.01–0.20)

NOTE: The higher the ratio the more complete is the 21-hydroxylation defect. From: CLEVELAND, W. W. and MIGEON, C. J. *Am. J. Dis. Child.*, 100: 493, 1960.[18]

pregnanetriol and 17-hydroxypregnanolone. The ratio of the pregnane compounds to the Porter-Silber chromogens can be used as a measurement of degree of the 21-hydroxylation defect.

Cleveland et al.[18] carried out the SU-4885 block in ten parents, five patients and six adult control subjects. Nine of the parents did not respond differently from normal control subjects. The ratios pregnanes:17-OHCS indicating the degree of 21-OH defect are shown in Table IV; disappointingly these do not show any detectable defect in the heterozygotes. It is of interest, however, that one father had the abnormally high ratio of 6 which is in the range of the affected patients. We discovered later that this is the same father who responded with abnormally high pregnanetriol when studied under ACTH stimulation by Childs et al.[15] We wonder whether he might be a homozygote, but have no evidence of this. We are still left without any method for detecting most of the heterozygotes.

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The Metabolic Basis for Certain Disorders of the Thyroid Gland

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THE ONLY known function of the thyroid gland is to provide the body with suitable amounts of two hormones: thyroxine and 3-5-3′-triiodothyronine. In order to accomplish this end, the gland must synthesize, store and secrete these two amino acids. This is accomplished by means of a complex sequence of biochemical steps which are only partially understood at the present time. It may be accepted as a generality that any impairment in any of these steps will inevitably be met by an attempt at compensatory hyperplasia of the gland through the well known feed-back control system involving the anterior pituitary gland. Compensation may be successful, incomplete or quite impossible.

It is the purpose of this study to review the central findings in the recognized categories of patients in which some degree of definition has been given to inherited defects which impair the function of the gland and result in a measure of hypothyroidism and glandular hyperplasia. More detailed reviews than is possible here may be found elsewhere.[1,2]

THE IODIDE-TRAPPING DEFECT

The first step in the synthesis of the thyroid hormones is the selective accumulation of inorganic iodide by the epithelial cells of the thyroid gland. Proper function of this iodide pump requires oxidative phosphorylation and is impaired by a number of metabolic poisons. A single patient has been recognized in whom this metabolic event does not take place.[3]

A fifteen year old schoolboy was admitted for study because of a goiter (Fig. 1). His early development was slow. An umbilical hernia was repaired at age three. When he was eight years old a diagnosis of hypothyroidism was made and thyroid medication was begun. His growth rate immediately increased, and his performance in school improved dramatically. Several months before the boy's admission to the hospital his mother noted a small mass in his neck which she attributed to the thyroid medication, and accordingly discontinued it. There was a prompt acceleration in the growth rate of the mass in the neck, but the patient became fatigued, lethargic and gained more weight. Detailed family history disclosed that the patient was a product of a marriage between first cousins and that other consanguineous marriages had taken place among his forebears. His father had a small goiter; his only sister was entirely normal. Examination disclosed evidence of mild hypothyroidism, but was otherwise within normal limits. There was a large, symmetrical goiter.

When it was found that the radioactive iodine uptake by the thyroid reached a maximal value of only 11 per cent, the possibility was entertained that the primary defect in the function of this gland might be impaired iodide trapping. In order to test this hypothesis, the ability of the salivary glands and of the gastric mucosa to concentrate iodide was tested on the surmise that these structures, also derived from the primitive gut, might share in the same impairment as the thyroid. Normally iodide is highly concentrated in the saliva and gastric fluid. It was found that there was no selective concentration of iodide by either the salivary glands or the gastric mucosa. In addition,

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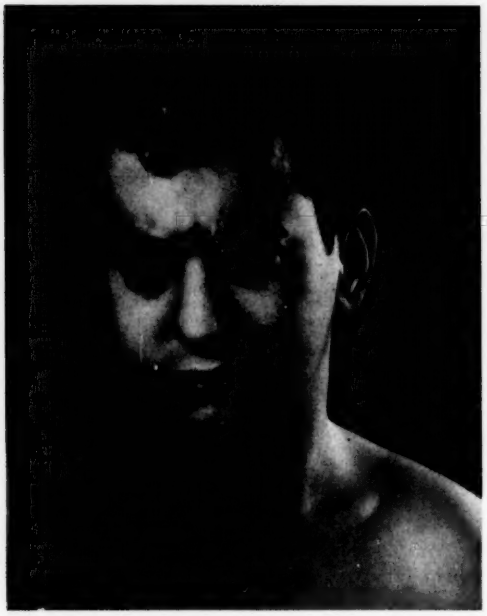

FIG. 1. A fifteen year old boy with hypothyroidism and goiter whose thyroid gland is unable to concentrate iodide.

it was found that the salivary glands also failed to concentrate thicoyanate, whereas normally this substance is concentrated approximately tenfold in the saliva. Thiocyanate also competes for iodide in the thyroid. Additional evidence was sought from studies on slices of thyroid tissue removed at biopsy. There was essentially no concentration of iodide by these slices *in vitro* under conditions which normally result in good uptake of iodide, nor were appreciable quantities of organic iodinated amino acids produced.

The final evidence derived from the hypothesis that if the only defect in the thyroid gland of this subject was impaired iodide trapping, then hormonogenesis should proceed at a normal rate if sufficient iodide could be provided by simple diffusion into the gland. Accordingly, the patient was given approximately twenty times the normal daily intake of iodine. On this regimen the basal metabolic rate rose within six weeks from minus 39 per cent to plus 8 per cent, all evidence of hypothyroidism vanished, and the thyroid diminished in size until, at the time of the last examination, it was barely palpable.

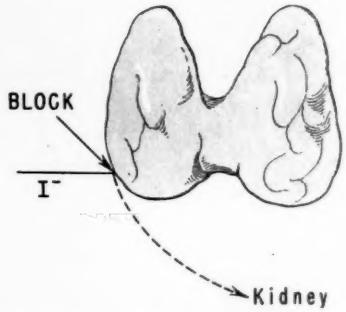

FIG. 2. Diagram indicating that the mechanism within the thyroid gland for concentrating iodide is absent and the circulating blood iodide is excreted by the kidneys.

The iodide pump of the thyroid gland undoubtedly requires the participation of many factors, including a supply of phosphate bond energy. The intimate details of the process are entirely obscure, as they are in all membrane transport systems. The finding that the potassium pump is necessary for the membrane transport of glucose[4] and that it is impaired by strophanthidine, and that strophanthidine also impairs the iodide pump,[5] suggests that the potassium pump is a necessary component in the iodide pump. This possibility merits investigation. In any event, the proximal explanation for the defect in this patient

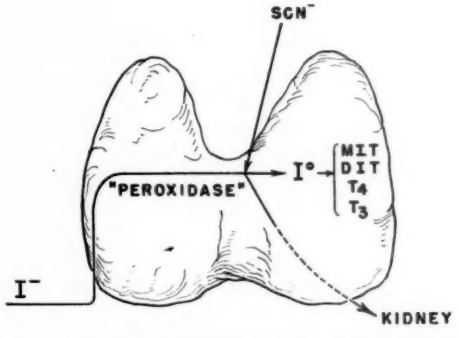

FIG. 3. Diagram indicating the type of hypothyroidism with goiter characterized by failure of organification of iodine. Thiocyanate causes a discharge of trapped iodide from the gland, which is then excreted in the urine. MIT = monoiodotyrosine; DIT = diiodothyronine; T_4 = thyroxine; T_3 = triiodothyronine.

is an absence of one of the components of the iodide pump and that his hypothyroidism and goiter were secondary thereto. The postulated defect is illustrated in Figure 2. It is of interest to note that had this patient subsisted in an area where the daily iodide intake was twenty times the normal value, his disease would never have been clinically evident. Thus, one may note the interaction of genetic endowment and environment in the production of clinical disease.

THE IODIDE ORGANIFICATION DEFECT

Iodide trapped in the thyroid gland must be oxidized to iodine in the process of iodination of tyrosyl residues in peptide linkage. An impairment in this step of thyroidal hormonogenesis was the first of the metabolic defects in familial goiter to be defined (Fig. 3).

The first patients were from a family of seven siblings, four of whom were imbecilic cretins with huge goiters.[6,7] Several consanguineous marriages were known to have taken place in their immediate forebears. Initial study disclosed that I^{131} was removed from the blood by the goiter with extreme rapidity and reached a plateau within one hour after the oral ingestion of the radioisotope. On the surmise that this iodide might be held only as inorganic iodide, the patients were given oral doses of thiocyanate. This substance will cause the discharge from the thyroid gland of inorganic trapped iodide but not of organically bound iodine. Administration of thiocyanate to these patients caused a precipitous discharge of the labeled iodine from the gland and its appearance in the blood and urine. The same effect may be obtained in these patients by administration of perchlorate, another substance which causes the release of inorganic iodine from the thyroid gland.

Further study confirmed the initial hypothesis. Observations on the kinetics of iodine metabolism in these patients indicated that the rate of disappearance of labeled iodine from the gland and its excretion in the urine was entirely consistent with a free exchange of all labeled iodine in the gland with that in the perfusing blood. This would not have been true if part or all of the labeled iodine in the gland had been in organic combination. Further, chemical analysis of biopsy specimens obtained after administration of I^{131} indicated that the only labeled iodine in the gland was inorganic. Chemical analysis likewise indicated the presence of extremely minute amounts of iodine.

A number of other patients with the complete defect in organic binding of iodide have been described. Careful study of the thyroid of one of these patients by Haddad and Sidbury[8] has shown that there is impaired organification of iodide *in vitro* in tissue slices. These investigators are of the opinion that the defect is an absence of the specific thyroid iodide peroxidase.

Recently a group of patients with familial goiter and nerve deafness, with or without mild hypothyroidism, have been studied and the hypothesis advanced that they belong to the same category of patients as those described in this group. The evidence has been that thiocyanate or perchlorate administered within one-half to one hour after ingestion of radioiodide causes a significant discharge of the labeled iodine from the gland. While it is entirely possible that these patients have a related but less severe defect, the possibility has not yet been excluded that the kind of discharge induced in these glands by thiocyanate or perchlorate, just as in patients with Hashimoto's thyroiditis under similar conditions, may represent a phenomenon common to many kinds of thyroid hyperplasia. Thus, iodide organification may be a rate-limiting step allowing for a build-up in the quantity of inorganic iodide in the gland which can be discharged with perchlorate. It is not yet proved with certainty that these patients suffer from specific impairment in iodide organification in a way identical with that in the patients of this group. This problem deserves further study and control.

THE IODOTYROSYL COUPLING DEFECT

Iodotyrosyl residues in peptide linkage in the thyroglobulin molecule are coupled and an alanine side chain extruded to form iodothyronines. The steric arrangements which are necessary to permit this coupling have not

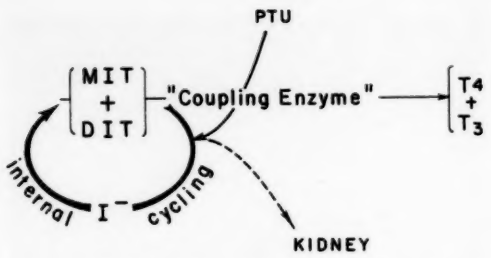

FIG. 4. Diagram illustrating the presumed defect in patients in whom coupling of iodotyrosines into iodothyronines fails. Propylthiouracil, by blocking the utilization of iodide, interrupts the internal recycling of iodide, and the iodide appears in considerable excess in the urine.

yet been defined. Indeed, almost nothing is known about the amino acid structure of thyroglobulin. Presently available information regarding N-terminal amino acid analysis is in conflict, but it seems clear that by specific peptidase action on thyroglobulin short chain peptides are produced, some of which are iodinated and most not.[9] Analysis of amino acid structure around the labeled nuclei in thyroglobulin would appear to be a most promising area of inquiry.

A number of patients have been described who appear to have an impairment in the coupling reaction. It has been surmised that the coupling of the iodotyrosyl residues is enzymatically controlled. There is no evidence whatsoever to support this hypothesis. The existence of such an enzyme is only supported by the fact that most, if not all, of the significant chemical processes of the body are enzymatically controlled.

Two sisters, whose immediate forebears included at least three intermarriages, were studied because of clinical hypothyroidism and large goiters.[7] Although there was clinical evidence of hypothyroidism extending over many years of careful clinical observation, at the time of study the determination of the protein-bound iodine concentration of the serum was within normal limits, as were the basal metabolic rates. In both, however, the radioiodine uptake on many occasions was nearly 100 per cent, and peak values were reached within an hour or two after oral administration. Thiocyanate failed to discharge the labeled iodine from the thyroid gland, but when these patients were given an antithyroid drug, such as propylthiouracil, there was an immediate accelerated rate of discharge of labeled iodine from the glands and the appearance of large amounts of labeled iodine in the urine. Calculation of the amount of thyroid hormone which this might represent indicated that, had it been calorigenically effective, these patients would have been very hyperthyroid, and the protein-bound iodine concentrations in their serum would have been high. The findings were consistent with an impairment in iodotyrosyl coupling in the gland and a resulting hypertrophy of the internal recycling of iodine. This internal recycling of iodide by deiodination of iodotyrosines has been well described and its specific enzyme, an iodotyrosyl dehalogenase, characterized[10] (Fig. 4).

Confirmation of the hypothesis was obtained when it was found that the gland of one of these patients, which was removed because of tracheal compression, contained large amounts of iodotyrosyl residues but scarcely detectable amounts of thyroxine or triiodothyronine.

THE DEIODINASE DEFECT

In the normal thyroid gland when thyroglobulin is degraded mono- and diiodotyrosine are released. These two amino acids are deiodinated by a potent dehalogenase present in the thyroid gland, liver, kidney and elsewhere. The iodide released in this process is reutilized as just described within the thyroid gland for further synthesis of thyroid hormone. A number of patients are now recognized who have familial hypothyroidism and goiter because this enzyme action is absent.[11–13]

The first of these patients studied was a twenty-seven year old man with life-long hypothyroidism and a goiter which had been observed at birth. He came to attention because of recent accelerated growth in the size of the gland. There was no known intermarriage in his family, but his brother was severely hypothyroid, mentally retarded, and had a large goiter. Radioactive iodine, administered orally, accumulated with great

rapidity in his thyroid, but after reaching a peak at seventy minutes, the labeled iodine rapidly left the gland. This event was accompanied by the appearance in the peripheral blood of iodinated components which proved upon chromatographic analysis to be mono- and diiodotyrosine and conjugates of these. Labeled mono- and diiodotyrosine also appeared in the urine. These substances do not normally appear either in the blood or urine, and if they are administered intravenously, they are rapidly deiodinated and appear almost quantitatively in the urine as iodide.

It was postulated that the reason for the metabolic anomaly was an absence of the dehalogenase in thyroid and in all other cells in the body (Fig. 5). It was then found that intravenously administered labeled diiodotyrosine was excreted almost quantitatively unchanged in the urine of this patient. When tissue slices became available at the time of surgery, no deiodinating ability was observed, whereas thyroid slices from normal thyroid glands and those with nodular goiter readily and rapidly deiodinate mono- and diiodotyrosine.

A number of other patients have been studied who have an identical disorder. The most thorough investigation of the appearance of the disease in families is that of McGirr[14] who observed a number of patients with this disorder among a group of Scottish tinkers, an itinerant and sequested group of people from Scotland, among whom intermarriage is extraordinarily common.

The hypothesis that this disease can be attributable entirely to an absence of dehalogenase has been attacked on the grounds that such a defect would not account for the virtual absence of thyroxine in the thyroids and in the peripheral blood of these patients. The explanation has been offered that a constant leak of iodinated amino acids from the thyroid causes the development of a vicious cycle of hyperplasia and increased secretion of iodotyrosines into the perfusing blood. If the hypothesis is correct, and if a single defect accounts for the findings, then one might surmise that the administration of iodide far in excess of the normal daily intake would

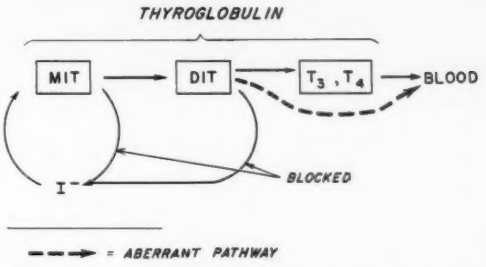

FIG. 5. The deiodinase defect. Reutilization of iodide made available by deiodination of mono- and diiodotyrosine is blocked. Mono- and diiodotyrosine, accordingly, escape into the blood and are excreted in the urine.

reverse the cycle and permit synthesis of a normal quantity of iodothyronines. This has recently been tested by Choufoer,[15] who found indeed that the administration of several milligrams daily of potassium iodide causes a reversal in the size of the goiter and a restoration of a normal metabolic state. The fundamental enzymatic defect, of course, remains, but its metabolic consequences upon thyroxine synthesis are negated by this maneuver.

ABNORMAL SERUM IODOPROTEIN

The normal secretion products of the thyroid gland are thyroxine and triiodothyronine. These are loosely attached to protein components in the peripheral blood and are readily extractable from the peripheral blood by butanol if it is acidified. From time to time the suggestion has been made that an abnormal iodoprotein has appeared in the peripheral blood of patients with nodular goiter. My first experience with this component was on two samples of blood received from Dr. Edward McGirr,[2] who obtained them from two sisters with hypothyroidism and huge vascular goiters. It was found that only 50 to 60 per cent of the labeled iodine in the blood of these subjects was extractable into butanol after acidification whereas normally 90 to 98 per cent is readily extracted. Recently a patient with severe retardation has been described who only had a nonbutanol extractable iodinated component in the peripheral blood.[16]

Something is known of the nature of this component. It has the electrophoretic mo-

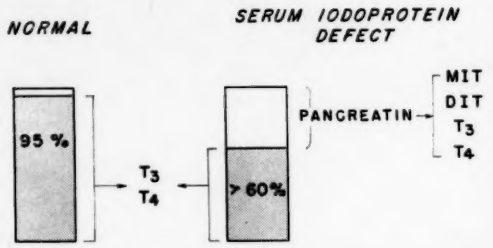

FIG. 6. Butanol-entractability of serum iodine. The labeled iodine in the blood of patients with the butanol extractable serum iodine component fails to enter the butanol phase. This component is hydrolyzed into the usual iodinated amino acids.

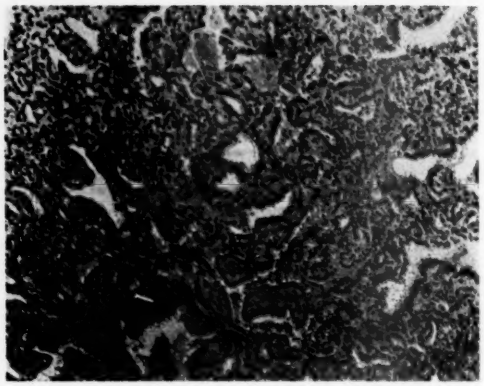

FIG. 7. The type of hyperplasia seen in the thyroid gland of patients with complete blocks in hormonogenesis.

bility of albumin. Its sedimentation constant is low. It is not normal thyroglobulin, but it can be hydrolyzed by ordinary proteolytic enzymes to yield mono- and diiodotyrosine and thyroxine, and after treatment with crude trypsin, the labeled iodine can be completely extracted into butanol (Fig. 6). What is not known is the reason for its appearance in the peripheral blood. One might speculate that it is an abnormal thyroglobulin which arises from aberrant or abnormal thyroglobulin synthesis and spills into the peripheral blood. Alternatively, one might suspect that it enters the peripheral blood because of impaired or incomplete proteolysis of normal thyroglobulin. One cannot escape the possibility that abnormal thyroglobulins exist, much as abnormal hemoglobins exist. Thus, structural abnormalities, by impeding normal proteolysis, or because the abnormally structured thyroglobulin is synthesized less rapidly than normally, impair delivery of the thyroid hormone. This hypothesis cannot be fully answered until the technics which have been so fruitful in the study of the abnormal hemoglobins have been applied to thyroglobulin.

COMMENTS

One might anticipate with considerable confidence that in the course of time other metabolic abnormalities will be described and characterized which interfere with the synthesis and delivery of the thyroid hormones. One might further anticipate that the groups which have been described will in time be subdivided, since at least in certain cases the same result can probably be achieved by interference with any one of several different essential components in one particular stage in iodine metabolism. Furthermore, groups may be subdivided in terms of severity of defect, because it is entirely possible that there may be gentic changes of varying degree in the same enzyme, each of which has its own effect on the specific activity of the enzyme.

An important consideration is the relationship of these defects to the larger problem of sporadic and endemic goiter. It is of interest in this regard that thyroid disease has been found in close relatives of patients with every one of the types of thyroid disease mentioned herein. Of patients with the dehalogenase defect, several relatives with thyroid disease were actually shown to be euthyroid and goitrous and to have the same defect to a limited degree as that exhibited by the probands. It seems probable that as methods are developed for detecting the heterozygous state, at least some patients with nodular or sporadic goiter will prove to be heterozygous for specific defects for which the severely affected patients with familial goiter and cretinism are homozygous.

Large amounts of iodide fed to patients with the dehalogenase defect and to the patient with the iodide trapping defect enabled metabolic compensation to be restored. Thus,

in some patients, iodide deprivation might greatly increase the hazard of a metabolic defect which, given normal amounts of iodide, would be harmless and without clinical implication. This hypothesis needs to be tested in various areas of the world where patients with severe endemic goiter can still be studied.

Many of the patients with simple or nodular goiter may have underlying defects identical with those described herein. The complete diagnosis of one of these patients should ideally include a thorough testing for each known defect. Unfortunately methods are not yet available for ascertaining with certainty the heterozygous state of any of these defects.

Finally, the role of these defects in thyroid neoplasia may be considered. The glands are intensely hyperplastic (Fig. 7). All the histologic characteristics of invasive neoplasia have developed in one of our patients. Thus, it seems possible that intense tissue hyperplasia, arising secondarily because of a metabolic block can lead to malignant change.

SUMMARY

At least five metabolically distinct disorders of the thyroid gland are now recognized which impair the function of the gland and lead to hypothyroidism and goiter.

The patients which have been described in each group are probably homozygous for a recessive gene which controls the synthesis of an enzyme necessary for a specific step in thyroidal hormonogenesis.

Relatives of these patients often have goiter but are otherwise normal. They are presumed to be heterozygous for the defect for which the probands are homozygous.

The quantity of iodine in the diet in some cases determines the impact of the genetically determined defect on the patient.

ACKNOWLEDGMENT

I wish to express my appreciation to the Honorary Editors of the *Proceedings of the Royal Society of Medicine* for permission to include in this paper data and Figures 2 through 7 which appeared in their journal. (STANBURY, J. B. *Proc. Roy. Soc. Med.*, 53:904, 1960.)

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Biochemical Genetics as Illustrated by Hereditary Galactosemia

HERMAN M. KALCKAR, M.D., PH.D.*

THE mere existence and biologic distribution of the sugar, galactose, is puzzling especially from the standpoint of an evolutionist. The difference between glucose and galactose is confined to the carbon atom number 4 (Fig. 1). The transition from glucoside to galactoside is essentially a kind of racemization between two configurations: the 4-hydroxyl in glucose occupies the equatorial position, whereas in galactose it occupies the axial position as related to the plane of the ring (Fig. 1).

It would be interesting to go deeper into the evolutionary aspects of galactose metabolism—the fact that the central nervous system and the milk gland have chosen to use galactose as one of their matrix elements. What could the positive vectors be? As will appear from the following discussion, galactose is not a necessary component of the diet of the newborn. Yet, we are not in a position to deny the possibility that given certain external influences the fitness of survival and reproduction may be influenced significantly by the presence of galactose in the diet. It is known, for instance, that the intestinal bacterial flora of the newborn animal is altered when lactose is replaced by sucrose. Nevertheless with only a few exceptions can we say that galactose is required by the living cell, insofar as this sugar can be made from glucose and glucose metabolites. As we shall see, we even have organisms for which the administration of galactose brings about serious disturbances.

Because of the complexity of galactose metabolism in higher animals, it is necessary to focus some attention on a particular chapter of carbohydrate metabolism and to introduce the various intermediary compounds which have been described in galactose metabolism. We are dealing here not only with free galactose or phosphorylated galactose but also with nucleotide hexoses, uridine-diphosphoglucose (URPPG) and uridine-diphosphogalactose (URPPGal). This important discovery we owe to Leloir.[1,2]

There are three major steps in the Leloir pathway, catalyzed by (1) Galactokinase: Gal + ATP → PGal + ADP; (2) Gal-1-P uridyl transferase: PGal + URPPG ⇄ PG + URPPGal; and (3) UDPGal 4-epimerase: URPPGal ⇄ URPPG.

The abbreviations used in this equation are meant to facilitate the understanding of mechanisms. Thus, galactose-1-phosphate is abbreviated to PGal in order to illustrate that the transferase catalyzes the "trading" of PG (glucose-1-phosphate) for PGal in the uridine nucleotide.

In animals, the enzymes catalyzing these reactions are largely or exclusively constitutive but unequally distributed among various tissues. In microorganisms, these enzymes are largely adaptive, i.e., they require galactose as inducer.

The system serves two functions: (1) to enroll galactose as an energy source in general carbohydrate metabolism, i.e., conversion to glucose-6-phosphate, triosephosphate or pyruvate; and (2) biosynthesis of more or less complex galactosides (and also polyglucosides).

As mentioned, most organisms, and certainly

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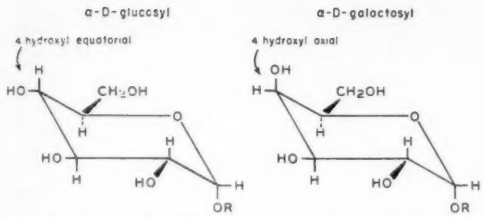

FIG. 1. Three-dimensional models of α-D-glucosyl and α-D-galactosyl compounds.

the higher animals, do not need any external supply of galactose for their growth and development. Whatever galactosides their tissues may contain can readily be made from glucose or from glucose metabolites through reaction 3, i.e., the 4-epimerase catalyzed reaction. A block in galactose metabolism is therefore apt to cause interference largely due to accumulation of intermediary products rather than on account of direct deficiency. As a general rule, jamming of intermediary products of a pathway is brought about by a block in any of the steps with the exception of the first one. This question will be dealt with more specifically later in this presentation.

CONGENITAL GALACTOSEMIA

An interesting type of a galactose-sensitive organism is the human subject afflicted with galactosemia. The disease in infants, congenital galactosemia, is characterized by specific tissue lesions (cataract, fatty degeneration in liver, retardation of brain function); by excretion of galactose in the urine; and, as shown by Schwarz and his co-workers,[3] by an accumulation of galactose-1-phosphate (Gal-1-P) in the red blood cells. These aberrations occur only if the affected subjects receive galactose. If a strictly galactose-free diet is imposed on the individual from birth, the organisms are practically insured against the development of specific galactosemic lesions. It is important here to bear in mind that in mammals the deposition of galactolipids in the central nervous system takes place exclusively after birth;[4] and yet, institution of a galactose-free diet provides practically complete protection against the development of cataract and feeblemindedness due to galactosemia.[5] This point is stressed here because it has been customary

TABLE I

Quantitative Comparison of Activities of Enzymes of Galactose Metabolism in Three Classes of Subjects

Subjects	Average Activities (μM conversion/hr./gm. cells, 37°c.)			
	Galactokinase	Gal-1-P Uridyl Transferase	UDP-Gal 4-Epimerase	PP-Uridyl Transferase
Nongalactosemic	0.10	4.8	0.32	10.0
Galactosemic	0.08	0.02	0.35	10.0
Galactosemic carriers	....	2.9	....	....

to refer to galactosemia as a block in 4-epimerase (Waldenase block). If this were so, postnatal galactose-free regimen would interfere with the deposition of brain galactolipids and, therefore, quite likely also with the development of normal intelligence.

An accumulation of Gal-1-P in the red cells of galactosemic subjects exposed to galactose might indicate the existence of an involved disturbance in the regulation of galactose metabolism somewhat analogous to diabetes mellitus, or alternatively a direct block in one of the enzymes of the Leloir system succeeding galactokinase. We preferred to focus our attention first on the enzyme Gal-1-P uridyl transferase, the existence of which had been predicted by Leloir[6] and which we observed a few years later in extracts of galactose-induced yeast.[7,8] We had developed specific enzymic methods[9,10] for characterizing most of these steps; we therefore considered ourselves reasonably well equipped to try our luck with the solution of the riddle of galactosemia. The enzymic technics permitted us to demonstrate that the defective enzyme in galactosemia is indeed Gal-1-P uridyl transferase; this was first demonstrated in lysates of erythrocytes (Table 1). The other enzymes of the Leloir pathway were present.[11–13] Although this observation did clarify a number of problems, we were far from a solution since we had raised some new ones. Following are some of the problems which, to some extent, were clarified by our observation.

In view of the foregoing discussion on the postnatal deposition of galactose in the brain,

it is particularly noteworthy that 4-epimerase (Waldenase) is present in about the same amount in galactosemic subjects as in nongalactosemic subjects.

The transferase defect could be demonstrated in blood from the umbilical cord of newborn infants.[14] This concurs, of course, with the fact that the disease is an inborn error.

By the use of C^{14}-labeled galactose, we were likewise able to demonstrate the same enzyme defect in liver biopsy samples from galactosemic subjects.[14] In one case, we wondered whether we might have encountered an incomplete enzyme defect (a "leaky mutant," as the microbiologists call it). In this galactosemic adult small amounts of transferase were found in his liver tissue but none in his hemolysates. I am inclined to think a related transferase may have developed in this subject which can incorporate Gal-l-P in a somewhat different way into nucleotide. Earlier we had described the existence of such a transferase in galactose-adapted yeast.[7,8] This uridyl transferase catalyzes a reaction between PGal and uridine triphosphate (URPPP) according to the equation:

$$\text{PGal} + \text{URPPP} \rightleftarrows \text{PP} + \text{URPPGal}$$

This reaction is apparently predominant in plants.[15] Moreover, Isselbacher[16] found the alternative transferase in rat liver. Hence, it is quite conceivable that the alternative transferase is present in small amounts in the liver of human subjects and may increase in adults.

PARTIAL ENZYMATIC DEFECTS

If congenital galactosemia is a hereditary enzyme defect, one would expect to find partial defects in parents of galactosemic children (heterozygotes). The first approach to this problem was made by Holzel and Komrower in 1955.[17] They used the galactose tolerance test as a criterion and found that either one or both the parents of galactosemic children had lower values in the tolerance test. The identification of one specific enzymic defect—the Gal-l-P uridyl transferase defect—encouraged us to try to devise a specific test for the detection of heterozygous carriers.

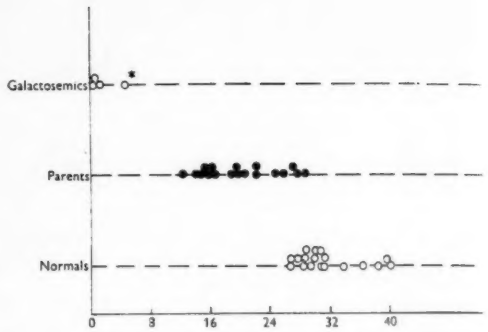

FIG. 2. The activity of Gal-l-P uridyl transferase expressed as μl. O_2 consumed per hour by 0.3 gm. of red cells from three different classes of subjects. (1) Nongalactosemic (mainly normal healthy persons); (2) persons suspected of being heterozygous with respect to the gene for the synthesis of the transferase since they have offspring with congenital galactosemia; and (3) corresponding homozygous affected persons, i.e., galactosemic. From: KIRKMAN, H. M. and BYNUM, E. *Ann. Human Genet.*, 23: 117, 1959.[18]

In order to make a technic available for this purpose, it would be most desirable to have a method with a built-in control which ensures that one is measuring rates in the range of zero order kinetics. Kirkman[18] used a very simple device, the old Barron-Engel'gardt-Warburg manometric determination of oxygen consumption in hemolysates (brought about by addition of methylene blue and metabolites). This method was so "tailored" as to fit the purpose of comparing transferase activities between normal subjects and suspected galactosemic carriers; it scores more than 20 points of measurements with high accuracy. The details of the method have been described elsewhere.[18]

The manometric assay showed[18,19] that about 80 per cent of the parents of the galactosemic subjects who were surveyed had enzyme titers which were more than 2 standard deviations below the mean of nongalactosemic subjects (Fig. 2). Kirkman is inclined to believe that if hemolysates from normal subjects, as well as hemolysates from brothers and sisters of suspected carriers, were assayed the manometric assay might identify practically all suspected carriers individually. To our knowledge, this is the first time that a method for measuring a partial enzyme defect stands a

chance of being used for such purposes, i.e., consultations of parents in matters concerning eugenics and preventive medicine.* With reference to the latter aspect, from Kirkman's distribution curve (Fig. 2) it will be evident that an appreciable fraction of carriers show enzyme titers below 50 per cent of the average of normal titers. This is of great importance in pregnancy, during which state the galactose tolerance is somewhat lowered for other reasons. If a pregnant mother, heterozygous with respect to the gene directing the synthesis of Gal-1-P uridyl transferase, carries a homozygous galactosemic fetus, excessive milk drinking during her pregnancy may expose the fetus to hazardous amounts of galactose. In a fraction of such cases an identification of the enzyme defect in the umbilical cord blood, such as we described earlier,[14] may therefore not be of much use with respect to dietary preventive measures. The preventive measures should have been used on the mother as well.

The incidence of hereditary galactosemia is not known. We have had thirty-five to forty cases under investigation: in all of our cases the transferase was affected; the other enzymes of the Leloir pathway were found to be unaffected. Our clinical colleagues can report numerous cases in which the disease was diagnosed incorrectly, the enzymic test established the true nature of the disease.[20]

That early diagnosis and institution of galactose-free diet is crucial is too well known a fact to warrant an elaboration here. If this is not done, permanent cataract, intelligence defects and liver cirrhosis will ensue. The biochemical basis for the tissue damage is unknown as yet. The discovery by Schwarz and his associates[3] that Gal-1-P accumulates in the erythrocytes of galactosemic subjects placed the spotlight on a most interesting problem: it has been found[21,22] that Gal-1-P inhibits phosphoglucomutase, i.e., the enzyme which catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate. As emphasized, especially by Najjar,[23] it seems worth considering this inhibition as being responsible for the development of some of the tissue damage characteristic in galactosemia.

In order to learn more about the genotypic and phenotypic characteristics of hereditary blocks in Gal-1-P uridyl transferase we have extended our search to microorganisms. It has been known for some time, mainly through the work of the Lederbergs,[24,25,28] that certain K_{12} strains of Escherichia coli are unable to metabolize galactose and are also unable to grow on galactose as the sole carbon source,

* A few methodologic problems which need clarification are discussed briefly here. Kirkman emphasizes that the manometric method gives about 50 per cent of the maximum values which can be obtained by the two-step enzymic method designed for determining initial rates. This design was performed as follows:[34] Small amounts of hemolysates (0.05 ml. of packed erythrocytes) were incubated at 37°c. for brief periods of time (e.g., five and ten minutes) with relatively high concentrations of reactants (0.17 μM of URPPG and 0.85 μM of Gal-1-P were used). If Tris buffer is used the rates of transferase in hemolysates from normal subjects (or more correctly, homozygous nongalactosemic subjects) ranged from 3.5 to 5.8 μM/gm. cells hour.[34] In the presence of phosphate buffer (either at pH 7.3 or pH 8) the rates are consistently cut down to half. It is quite possible by the modified two-step enzymic method to find lowered rates in hemolysates from heterozygous carriers of galactosemia; we found values as low as 1 to 1.5 μM.[34] By the use of an almost identical set-up, Brettauer and his co-workers[36] recently obtained the following values: nongalactosemic subjects, 4.8 to 8.1 μM; carriers of galactosemia, 2.9 to 3.7 μM. These values are somewhat higher than ours. One may logically ask why not use the enzymic technic just outlined as the method in population studies, especially since the manometric method is best reproduced in phosphate buffer at pH 7.3, conditions which permit only half of the maximum rates which can be found in Tris buffer?

The reason for our choice of the manometric method is that it shows the investigator whether he is measuring initial rates (zero order kinetics) or not, and this is the essential point in comparing enzyme rates from homozygotes and heterozygotes. The enzymic method as used by Brettauer and his co-workers,[36] i.e., scoring 1 point, does not give such a guarantee and should, therefore, not be used as the sole method "to detect parents who are likely to give birth to galactosemics."[36] Hsia et al.[37] failed to provide convincing evidence for enzyme defects in heterozygotes because they used, against our warnings, a diagnostic method which we had designed exclusively for detecting galactosemic patients.[4] It is obvious that considerations of simple kinetics must be given due respect when partial enzyme defects in heterozygotes are under study. The manometric method would in general be the reliable guide at the present time.

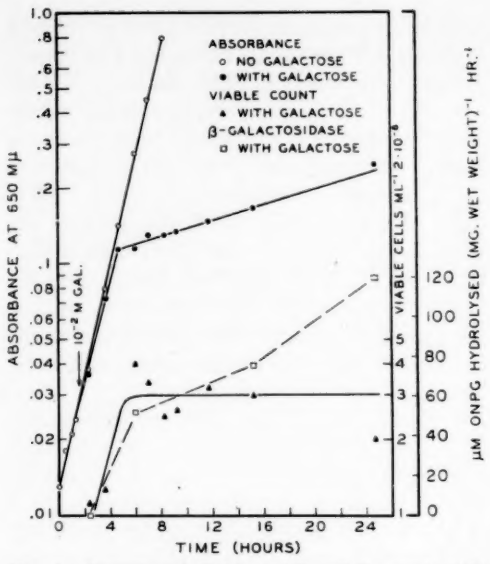

FIG. 3. Growth and division of E. coli K_{12} strain 3104 at 37°c. under aerobic conditions. Growth as well as viable counts plotted logarithmically. The activity of β-galactosidase (on a cellular basis) is plotted linearly and expressed as micromoles of orthonitrophenyl galactoside hydrolyzed per hour per micrograms toluenized cells (wet weight). The β-galactosidase activity of the cells which were grown in medium without galactose was too low to record on this graph (less than 1 μM/mg./hour). The β-galactosidase activity in the medium of nontoluenized cells grown with or without galactose is negligible (less than 1 μM/mg./hour). From: YARMOLINSKY, M., KALCKAR, H. M., WIESMEYER, H. and JORDAN, E. *Proc. Nat. Acad. Sc.*, 45: 1786, 1959.[30]

so-called gal-mutants.[24,25] The many gal-mutants had so far only been characterized genotypically. The challenge was to try to characterize them phenotypically and more specifically with respect to the supposedly primary gene products, the enzymes.

Kurahashi[26] in our group first undertook to apply the enzymic technics used in the identification of the defect in hereditary galactosemia to bacterial mutants. It soon became apparent that there were at least four genotypically different mutants which were phenotypically identical, i.e., they all had a practically complete defect in transferase. Likewise, there were three different mutants (different "mutons" in same "cistron"[27]) which phenotypically could be characterized as "galactokinase-less." Finally, there were various types of single mutants[28] "leaky" with respect to the two enzymes mentioned (i.e., greatly lowered activity as compared with the wild type) and more or less completely defective with respect to the 4-epimerase.[29]

Characteristic for the genotypes, which are unable to synthesize Gal-1-P uridyl transferase, is a phenomenon which we call "galactose-induced sensitivity" because the cells react like cells in an unbalanced state only when galactose permease and galactokinase are induced.[26,29,30] Under these conditions, Gal-1-P accumulates in large amounts just as in the afflicted children referred to previously. In most of the E. coli strains with which we have been working (Lederberg's K_{12} galactose mutants), the abnormal state could be called "galactose-induced bacteriostasis" because the cells do not lyse and remain viable[30] (see Fig. 3). The lack of lysis is borne out not only by the fact that turbidity and viable cell count remain constant but also by the fact that the medium does not contain more than traces of enzymes such as β-galactosidase. This should be emphasized because Fukasawa and Nikaido[31a] have recently described galactose-negative mutants in which induction by galactose brings about lysis and death, unless the cells are grown in hypertonic medium (*cf.* also Ushiba and Kitasato[31b]) in which case cells without cell walls (protoplasts) are formed. We have shown,[29] as well as Fukasawa and Nikaido,[32] that these "galacto-lytic" strains have a single defect in 4-epimerase.

The galactose-sensitive strains (either galactostatic or galactolytic) can undergo mutation. If grown in glycerol ammonia mineral medium in the presence of galactose, there is a strong selection either for reversions to galactose-positives or further mutation to galactose-negatives with an additional block early in the pathway. In this way we have obtained double mutants.

The relevance of these microbiologic studies to the problems of galactosemia in man is obvious. In the transferase-less bacterial mutants galactose-l-phosphate accumulates during the induced galactostasis; UDPGlucose levels tend to go down. In the galactolytic

state, as well as just prior to this state, there is a striking accumulation of UDPGalactose.[33]

The nature of the genetic block in terms of protein (i.e., absence of protein or production of proteins which crossreact with antibodies against transferase but are catalytically inactive) is under study.

At the present time several groups in America have had success in cultivating skin fibroblasts from human subjects. I have voiced the opinion that hereditary galactosemia at present is one of the few inborn errors of metabolism which can be studied in tissue cultures.[34] This is due to the following circumstances: (1) Galactosemia has been identified as a defect in a particular enzyme which is relatively well defined, transferase (Gal-1-P/G-1-P uridyl transferase). (2) The transferase defect is not confined to a single type of cell but has been demonstrated in any cell type which shows galactose metabolism. (3) Mutations to reversions or transformations with DNA from transferase-negative to transferase-positive might be readily detected and selected because of the toxic effect of galactose toward the transferase-negative cells.

Krooth and Weinberg[35] have recently been able to demonstrate the defect in galactose metabolism in fibroblast cultures from galactosemic subjects. Such cultures are unable to use galactose as a hexose source and unable to form carbon dioxide from galactose. The latter study was performed with C^{14}-labeled galactose, collecting radioactive carbon dioxide. This seems to constitute a highly sensitive method. In our laboratory we have found a defect of transferase in sonicates from fibroblast cultures from galactosemic subjects. Enzyme studies on galactosemia in tissue cultures are only the beginning. Enzyme defects in glucose-6-phosphate dehydrogenase or in phosphatase should lend themselves, too, to fundamental studies.

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Glycogen Storage Disease

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GLYCOGENOSIS, the accumulation of increased amounts of glycogen in the organs and tissues of the body, is a problem which has attracted a number of investigators. Since the various aspects of the glycogen storage diseases have been extensively reviewed,[1–7] this discussion will be limited primarily to a consideration of the cases studied in the Cori laboratory during the last decade. We are indebted to the scores of physicians throughout the United States and abroad who have provided tissue samples and clinical resumés.

INTRODUCTION

The reserve carbohydrate of the mammalian organism, glycogen, is a relatively simple molecule being composed of glucose units linked by α-1,4 and α-1,6 bonds. However, the mechanism of the formation and breakdown of this simple polysaccharide and the factors which influence its anabolism and catabolism are complex, and it is in this area that an explanation for the excessive accumulation of glycogen must be sought. Figure 1 is a model of a segment of a glycogen molecule. R designates the free aldehydic reducing group at carbon 1 (C-1), all other C-1 carbonyl groups being linked to a neighboring glucose unit. The α-1,4 bond can be made or broken by the enzyme phosphorylase,[8] its reversible reaction being represented by the equation:

$$\text{glucosyl chain}_{(n)} + \text{glucose-1-P} \rightleftharpoons \text{glucosyl chain}_{(n+1)} + \text{P}_{\text{inorganic}}$$

Pre-existing maltosidic chains in glycogen may also be lengthened by transfer of glucose from uridine diphosphoglucose (UDPG) to the chain, a reaction first described by Leloir and Cardini,[9] and subsequently investigated by Leloir et al.,[10] Robbins et al.,[11] Villar-Palasi and Larner,[12] and Hauk and Brown.[13] The bond formed is also α-1,4. The UDPG glycogen transglucosylase reaction is essentially irreversible, and hence is of importance only in the anabolic sense.

Two separate enzymes have been implicated in the formation and rupture of the α-1,6 bond. Hestrin[14] demonstrated that glycogen was incompletely digested by highly recrystallized phosphorylase, the product formed by phosphorylase being termed a limit dextrin. Subsequently, an enzyme, amylo-1,6-glucosidase, was isolated and shown to form free glucose by hydrolytically cleaving the α-1,6 bond of the glucose unit located at the branch point.[15] Prior action by phosphorylase to expose this 1,6-linked glucose unit is a prerequisite for the action of amylo-1,6-glucosidase. After glucosidase action on the limit dextrin, the molecule is again susceptible to phosphorylase degradation until the second tier glucose branch points are reached, when glucosidase must again act. By the use of these two enzymes stepwise, serial degradations of polysaccharides isolated from patients with glycogenosis have been made and details of their structures have been elucidated.[16] Usually, a measure of the proportion of glucose units located beyond the outermost tier of branch points as evidenced by the per cent digestion of the isolated glycogen by purified phosphorylase free of amylo-1,6-glucosidase is

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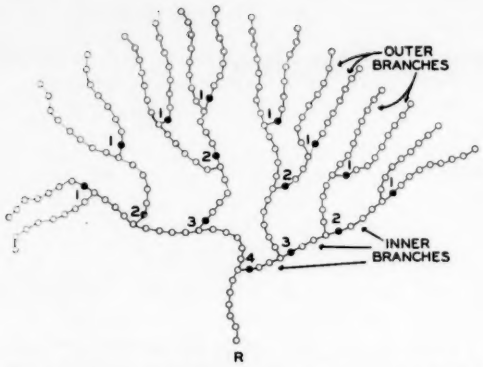

FIG. 1. Segment of glycogen molecule. O,α-1,4 linked glucose units. ●,α-1,6 linked glucose units susceptible to hydrolytic cleavage by amylo-1,6-glucosidase (see text).

sufficient to give information about the nature of the polysaccharide accumulated in cases of glycogen storage disease.

Larner and Schliselfeld[17] have shown a limited reversibility of amylo-1,6-glucosidase. Incubation of this enzyme in the presence of C^{14} labeled glucose and polysaccharide resulted in a product which had incorporated up to 0.1 per cent of the radioactivity. As will be seen, this limited incorporation of glucose has been utilized by Hers[5] as a method for assaying for amylo-1,6-glucosidase activity in tissue extracts.

Amylo-1,4→1,6-transglucosidase, the enzyme responsible for the introduction of branch points into the glycogen molecule, was studied by Larner.[18] He showed that after lengthening the exposed chains of glycogen by incubation with C^{14} glucose-1-phosphate and phosphorylase, some of the labeled glucose residues, originally linked α-1,4- became linked α-1,6- when the polysaccharide was subsequently treated with a crude enzyme from liver. Similar results could be obtained with muscle extracts. These observations were interpreted as evidence for the action of a "branching" enzyme which formed new outer branches by the transfer of a segment of a maltosidic chain to C-6 of an acceptor glucose residue. The length of the segment transferred is unknown.

GLYCOGEN STORAGE DISEASE

Type I (Glucose-6-Phosphatase Deficiency)

The first type of glycogen storage to be described clinically was also the first to be explicable on a biochemical basis. In 1928 van Creveld[19] described a syndrome of disturbed carbohydrate metabolism in an eight year old boy with hepatomegaly. Then in 1929 von Gierke,[20] published a description of the clinical and pathologic findings in two children with enlarged livers and kidneys which at autopsy were shown to have accumulated large amounts of glycogen. The disease became known as von Gierke's disease. It was not until 1952 that Cori[21] demonstrated that livers and kidneys from such patients were deficient in the enzyme glucose-6-phosphatase which is responsible for the liberation of glucose from glucose-6-phosphate. Since this enzyme is important in the maintenance of blood sugar in the normal liver, the loss of the enzyme accounts for the clinically observed tendency to hypoglycemia, the lack of glycemic response to

TABLE I
Type I Glycogenosis (Glucose-6-Phosphatase Deficiency)

No. of Cases	Glucose-6-Phosphatase (μg. P/100 mg./hr.)	% Glycogen in Liver	Patients with Sibling Data	No. of Children in Affected Families		
				Unaffected	Affected	
					Survival	Non-survival
10 (autopsy)	21 (8–39)	4–12	7	7		13
16 (biopsy)	39 (21–54)	5–18	12	16	17	4

TABLE II
Persistence of Low Glucose-6-Phosphatase Activity

Patient	Age at Biopsy	% Glycogen		Glucose-6-Phosphatase (μg. P/100 mg./hr.)	Epinephrine Response (mg. %)	
		Liver	Muscle		Fasting Blood Sugar	Rise
J. C.	3½ yr.	13.2	1.4	39	48	12
	5 yr.	6.3	1.7	52	42	28
	9 yr.	7.9	1.5	84	—	—
A. C.	4 mo.	8.8	0.5	36	45	5
	22 mo.	9.4	1.5	40	48	12
	6 yr.	7.1	1.3	74	—	—

epinephrine, and the failure of the appearance of blood glucose following the administration of galactose seen in this syndrome.

The assay for glucose-6-phosphatase activity consists in incubating for one hour at 30°c. a 1 ml. reaction mixture, 0.01 M in glucose-6-phosphate and 0.03 M in sodium citrate, pH 6.6 and containing a homogenate of liver equivalent to 5 to 20 mg. of tissue. The amount of inorganic phosphorus formed is measured in a trichloroacetic acid filtrate, and the results are expressed as micrograms of phosphate liberated from glucose-6-phosphate per 100 mg. liver per hour.

As will be seen in Table I, twenty-six patients with glycogen storage disease due to a deficiency of glucose-6-phosphatase have been studied in our laboratory. Autopsy material was available in ten, seven of whom died by one year of age, the other three survived until three, five and ten years of age. Data on siblings were not available in three of these ten cases, but of the thirteen siblings reported for the remaining seven, six died before the age of one year with clinical histories consistent with a diagnosis of glycogen storage disease.

Biopsy specimens were obtained from sixteen patients, eleven of them infants less than one year old, the others being two, three, three and a half, five and six years old. With the exception of a child who died at the age of eight years from an infection of the respiratory tract, the group includes children who are now from four to fourteen years old. The values of glucose-6-phosphatase activity and glycogen content are not significantly different from those in the autopsy group. A word should be said about the range given for the glycogen content of the liver. Usually, the amount found is from 7 to 10 per cent; the extremes represent single cases. In type I glycogenosis the level of apparent glucose-6-phosphatase activity is of the order of 5 to 10 per cent of normal.

Table II shows representative data from two cases selected because biopsy specimens were obtained from the brothers on three occasions.[4] The suggestion had been made earlier that the survival of patients with this type of glycogen storage disease was due to a delayed appearance of the phosphatase, an enzyme which is known to be formed late in fetal life.[22] However, in neither case was there a significant change in the measurable amount of glucose-6-phosphatase.

Of interest have been the studies by van Creveld[23,24] in which he has followed both his original patient of 1928 and a second one described in 1933. Both patients have become the parents of healthy children. The fasting hypoglycemia has disappeared but neither patient shows a response to epinephrine. However, the lack of response to epinephrine is not unique in type I glycogenosis, and, since the male patient has been reported to have muscles containing large quantities of glycogen[23] and to convert galactose to glucose,[24] this case may not belong to this category.

Type II (Generalized Glycogenosis)

In 1932 Bischoff,[25] Pompe[26] and Putschar[27] separately described fatalities in infants with

TABLE III
Type II (Generalized Glycogenosis)

Patient*	% Glycogen			Glucose-6-Phosphatase (μg. P/100 mg./hr.)	No. of Children in Family†
	Liver	Muscle	Heart		
D. B.	6	7	7	513	●●○○
D. C.	8	10	8	440	●○
C. D.	9	12	6	540	●●○
P. F.	5	11	11	500	●●○○○
D. K.	10	10	12	461	●●○
H. K.	6	12	8	266	●●○
P. W.	7	8	8	...	●●○○
W.	10	...	9	448	●●●○○
G.	...	12	...	...	●●

* Plus seven additional patients.
† ● Affected children, all of whom died.
○ Unaffected children.

cardiomegaly and diffuse deposition of glycogen in the heart. Whereas polysaccharide storage in the heart is so marked that the syndrome has been called the cardiac type, investigations of other tissues have disclosed widespread storage, so that "generalized glycogenosis" is a preferred term. Sixteen cases of generalized glycogenosis have been examined in our laboratory. All autopsies were performed on infants less than one year of age. The figures (rounded off to the nearest whole number) for glycogen content are given in Table III.

Values for glucose-6-phosphatase activity which approach the levels seen in normal livers are included for comparison with those obtained in type I glycogenosis. In the last column data on siblings are tabulated; the filled circles represent affected siblings all of whom died and the open circles represent unaffected children. Two (D. K. and H. K.) were siblings; in this family the incidence was two of three children. Despite the availability of autopsy tissue, all attempts to demonstrate an enzymatic lesion responsible for the accumulation of these large amounts of glycogen have so far proved fruitless. Isolated glycogens exhibit normal structure and physical chemical properties. Homogenates of generalized glycogenosis tissues appear to have significant activity when tested for phosphorylase, UDPG glycogen transglucosylase, amylo-1,6-glucosidase, hexokinase, phosphoglucomutase, fructose-1,6-diphosphatase and endogenous glycogen breakdown. The glycogenolytic function of the liver is preserved during life as evidenced by the lack of hypoglycemia and a normal response to epinephrine. The possibility that there is interference in the conversion of excess carbohydrate to fat remains to be investigated.

TABLE IV
Type III (Limit Dextrinosis)

Patient	% Glycogen		Isolated Glycogen % Split by Phosphorylase		Amylo-1,6-Glucosidase
	Liver	Muscle	Liver	Muscle	
C. B.	...	3.5	...	6	Not tested
C. B.	14	...	16	...	Absent
M. C.	17	...	18	...	Not tested
R. D.	15	4.3	16	6	Absent
W. D.	11	7.5	17	8	Not tested
B. F.*	8	8	10	11	Absent
C. H.	11	4.6	12	2	Not tested
B. J.	26	...	21	...	Not tested
K. J.	16	4.4	10	3	Not tested
K. K.	16	...	12	...	Absent
A. L.	14	2.2	19	8	Absent
C. L.	12	1	18	5	Absent
D. L.*	14	4	13	4	Absent
M. L.	17	5.4	17	3	Absent
V. M.	16	2.3	...	19	Not tested
T. P.	16	1	19	13	Not tested
R. T.*	13	...	13	...	Not tested
R. W.	...	4.3	...	11	Absent

* Autopsy.

Type III (Limit Dextrinosis)

The third type of glycogen storage disease was initially delineated by work performed in this laboratory. In 1952 it was reported that glycogen isolated from the tissues of a twelve and a half year old girl had an abnormal structure and actually resembled a limit dextrin.[16] That such a polysaccharide accumulates due to a deficiency of amylo-1,6-glucosidase, the debranching enzyme, was demonstrated a few years later.[28]

Table IV summarizes the eighteen cases of this type seen here and includes unpublished data. Three biopsy specimens were from infants six months old, the others were obtained from individuals two to thirty-two years of age. The extremely high content of liver glycogen and the range of values obtained for muscle glycogen should be noted. In particular, it should be emphasized that apparently normal glycogen levels of 1 to 2 per cent have been encountered in muscles derived from patients

with limit dextrinosis. Whereas glycogens isolated from normal subjects or from those with other types of glycogenoses are digested to the extent of 30 to 40 per cent by the action of phosphorylase free of amylo-1,6-glucosidase, the percentage of glucose units liberated from glycogens derived from patients with limit dextrinosis is significantly less, particularly from the muscle glycogens. In the nine cases in which the activity of amylo-1,6-glucosidase was investigated, values were determined which were at most 10 per cent that found for normal tissues. Multiple muscle samples were available in one patient (B. F.), one tissue having a glycogen content of 14 per cent. Amylo-1,6-glucosidase was lacking in three different muscles samples obtained from this patient, including the heart which had a glycogen content of 4.5 per cent. As previously reported[4] the only evidence for the familial nature of this type of glycogenosis rests with one family, W. D. and R. D., who are male and female siblings, and R. W., a maternal cousin.

The availability of highly purified amylo-1,6-glucosidase and a modification of the assay system proposed by Hers[5] gave the results reported in Table v. Reaction mixtures containing glycogen, homogenate equivalent to about 40 mg. tissue, sodium fluoride, potassium phosphate and glucose uniformly labeled with C^{14}, were incubated at 30°c. for several hours. The glycogen was then re-isolated, purified by repeated precipitation and plated for radioactivity measurement. Under these conditions the use of homogenates of liver or muscle from normal subjects or from subjects with glycogenoses other than limit dextrinoses results in reisolated glycogens containing 60 to 150 counts/minute/mg. glycogen. Only traces of radioactivity were present in glycogens re-isolated after incubation of homogenates of tissues from subjects with limit dextrinoses. In three separate experiments, the addition of amylo-1,6-glucosidase resulted in significant incorporation compared to the unsupplemented reaction mixtures incubated at the same time (Table v). Experiment C also demonstrates that the counts incorporated are proportional to time of incubation and quantity of added enzyme. The fact that the addition of amylo-1,6-glucosidase to such homogenates restores their ability to incorporate C^{14} glucose into glycogen confirms the absence of this enzyme in limit dextrinosis.

TABLE V

Effect of Addition of Purified Amylo-1,6-Glucosidase to Tissue Homogenates from Patients with Limit Dextrinosis

Experiment	C^{14} Glucose Incorporation*	
	Homogenate Alone	Homogenate Plus Added Amylo-1,6-Glucosidase
A	2.6	86
B	0.7	38
C	1.5	29 (4.5 μg. protein, 4 hr.)
		66 (18 μg. protein, 2 hr.)
		113 (18 μg. protein, 4 hr.)

* Values given in terms of counts per minute per milligram re-isolated glycogen following incubation as described in text.

Type IV Glycogenosis

Type IV glycogenosis is represented by a single case, that reported by Andersen.[29] The polysaccharide isolated from the liver at autopsy was shown[16] to have an abnormal structure more closely resembling amylopectin than glycogen. The relative paucity of branching was reflected in long outer chains (maximum absorption of the iodine complex, 530 mμ.) and a definite degree of crystallinity as revealed by an x-ray powder diagram. Type IV glycogenosis is presumed to be due to a relative deficiency of amylo-1,4→1,6-transglucosidase, the branching enzyme. A sibling is reported to have presented similar histological findings at autopsy.[29] No biochemical studies were made.

Type V Glycogenosis (Absence of Muscle Phosphorylase)

In 1959 two groups, Mommaerts et al.[30] and Schmid et al.[31a] published detailed studies of two separate cases of glycogen storage in muscle associated with an absence of phosphorylase. These will be classified as Type V. Both patients had muscle glycogen contents of

TABLE VI

Glycogen Content and Enzymatic Activities of Liver Homogenates in Hepatic Storage Disease

Patient	% Glycogen	Glucose-6-Phosphatase (μg. P/100 mg./hr.)	Phosphorylase (units/gm.)	Epinephrine (Δ mg. % blood sugar at 30 min.)
R. C.	6.2	650	8,830	None
W. J.	15.2	846	6,500	Good
B. M.	8.7	776	4,900	...
S. P.	14.5	500	6,250	44
B. K.	11.2	514	8,160	40
J. A.	15.9	548	1,000	48
R. A.	9.0	450	440	40
D. P.	11.7	328	500	5
W. O.	13.0	385	600	35
M. H.	15.3	188	1,150	Poor

3 to 4 per cent, and showed no lactic acid formation from endogenous glycogen. Homogenates of muscle were able to glycolyze added hexose phosphates at normal rates. The enzyme defect consisted in the specific absence of muscle phosphorylase. Isolated glycogen had a normal branched structure and was completely degraded by phosphorylase plus glucosidase. UDPG glycogen transglucosylase activity was demonstrated in extracts of muscles from both patients. This demonstration of UDPG glycogen transglucosylase and of glycogen in the absence of phosphorylase provides the best support for the idea of the importance of the UDPG mechanism in glycogen synthesis. That this type of glycogen storage disease is familial was recently demonstrated by Schmid and Hammaker.[31b]

Type VI Glycogenosis

As a final group, there are to be considered twenty-six patients in whom the disturbance seems to be confined to the liver. Both liver and muscle biopsy specimens were obtained in sixteen cases; in ten others only liver tissue was available The livers, which were all enlarged, contained 6 to 16 per cent glycogen of normal structure. Moderate to very high levels of glucose-6-phosphatase activity have been measured in all cases. The muscles, when examined, contained less than 2 per cent glycogen and this had normal structure. The younger children exhibited fasting hypoglycemia, eight of the biopsy specimens being from infants less than two years old.

In Table VI data are given for ten cases in which a measurement was made of liver phosphorylase activity. Those cases in which liver phosphorylase was diminished may constitute a group and belong in a separate category.[5] It should be pointed out, however, that liver phosphorylase is known to exist in active and inactive forms, and the enzymatic mechanisms for its inactivation and reactivation are complex and not wholly understood. Until a reproducible *in vitro* system is available for measuring *total* liver phosphorylase, the question of the role of this enzyme in the development of hepatic glycogen storage disease must remain to some extent unanswered. Epinephrine has been shown to have an effect in the enzymatic transformation of inactive liver phosphorylase to the active form.[32] Thus, in those patients with low liver phosphorylase in whom there was a good glycemic response (for example, J. A., R. A. and W. O.), higher levels of active phosphorylase may be present in the intact liver than that measured in the homogenates of the biopsy samples.

Glucose-6-phosphatase activity was measured in all twenty-six of the livers. M. H. is one of the nine patients showing values less that 300 μg. phosphorus per 100 mg. liver per hour. These patients might be considered to have a mild form of type I, particularly one (M. H.) who showed a poor response to epinephrine. However, the other eight with intermediate glucose-6-phosphatase levels showed a normal glycemic response to epinephrine.

Among the twenty-six children there are four pairs of siblings: three male pairs and one male and female pair. In addition, two other children have siblings with similar symptons. There have been no deaths reported in this group and one of the subjects is nearly thirty years old.

MULTIPLE LESIONS

Although one might expect that in any one family glycogenosis of only one type would occur, a few instances of what appear to be multiple lesions, presumably due to disturbance at more than one genetic locus, have been observed. One instance of a family with one

TABLE VII
Patients with Possible Multiple Lesions

Patient	Type	Glucose-6-Phosphatase (μg. P/100 mg./hr.)	Amylo-1,6-Glucosidase* (C./min./mg.)	% Glycogen		Isolated Glycogen (% Split by Phosphorylase)
				Liver	Muscle	
R. L.	I	21		18.5	2.1	30 (muscle)
A. L.	III	180	2.7	14.8	2.2	8 (muscle) 19 (liver)
C. B.	I-III	21	4.1	13.8	0.3	10 (liver)
D. M.	I-V†	53	60	6.4	0.3	37 (liver)

* See text and Table V for assay procedure.
† Muscle phosphorylase, 117–267 units per gm. to be compared with normal activities of 2,000 to 4,000 units per gm.

child with type I glycogenosis and another with type III (limit dextrinosis) was reported by Manners.[33,34] We have obtained tissue from another subject with limit dextrinosis whose sibling was reported to have died with type I disease. In addition, biopsy materials were available from still another pair of siblings with types I and III glycogenosis, as well as from two individual children each exhibiting possible double lesions. The data supporting these diagnoses are summarized in Table VII. A. L. and R. L. are siblings. The liver from one (R. L.) had a high glycogen content, the muscle glycogen a normal structure, 30 per cent of the glucose units being split by the action of phosphorylase free of amylo-1,6 glucosidase. A very low level of glucose-6-phosphatase was found in the liver. The other (A. L.) exhibited some deficiency in phosphatase, the value of 180 being significantly less than that normally found, but a more marked deficiency was apparent in amylo-1,6-glucosidase. That the polysaccharide isolated from the tissues of A. L. approached the limit dextrin structure was shown by the low percentage of glucose units split by phosphorylase acting alone. The liver from a three year old child (C. B.) had markedly diminished phosphatase and glucosidase activities. The polysaccharide isolated from the liver resembled a limit dextrin. Enzymatic assays on biopsy tissues from another subject (D. M.) revealed a decreased liver phosphatase and a decreased muscle phosphorylase. Considerable activity of UDPG glycogen transglucosylase was measured in the muscle. The isolated liver glycogen had normal structure.

Samples of muscle and liver glycogen isolated from a nine year old boy demonstrated to be deficient in glucose-6-phosphatase by Dr. K. Steinitz were sent to us for structural analysis. Both these glycogens had abnormally short outer chains. This child may be similar to C. B. in having no amylo-1,6-glucosidase as well as a deficiency in glucose-6-phosphatase.

The limit dextrinosis case (B. F., Table IV) in which there was a demonstrated lack of amylo-1,6-glucosidase might also have been a case of generalized glycogenosis. The liver glycogen content was lower and the muscle glycogen contents significantly higher than generally encountered in limit dextrinosis. Since no enzymatic lesion has been determined for generalized glycogenosis, the multiplicity of the defect could not be ascertained.

SUMMARY

Table VIII summarizes the distribution of the eighty-nine cases of glycogenosis from which tissues have been available. In three of the types a definite and specific enzymatic lesion has been determined. Type I, due to a deficiency of glucose-6-phosphatase; type III, a deficiency of the debranching enzyme, amylo-1,6-glucosidase; and type V, a lack of muscle phosphorylase. It is presumed that the branching enzyme, amylo-1,4→1,6-transglucosidase is diminished in type IV. Although the mechanism responsible for the accumulation of glycogen in type II is obscure, the defect

TABLE VIII
Summary of Patients Seen in the Cori Laboratory (1950–1960)

Type	No. of Patients	Patients with Sibling Data	No. of Children	
			Total	Affected
I	26	19	57	34
II	16	13	38	21
III	18	8	22	11
IV	1	1	2	2
V	2	1	1	1
VI	26	16	35	22

must be present in all tissues. It is possible that those patients in group VI who exhibit a diminished level of liver phosphorylase activity might be classified as a separate group; those with moderate levels of glucose-6-phosphatase might be considered to have a mild type I glycogenosis.

Although the more lethal the type of glycogenosis the more information is available concerning siblings, the familial nature of the diseases is apparent. That the genetic loci for determining and controlling the formation of glucose-6-phosphatase and amylo-1,6-glucosidase are closely related is suggested by the association of type I and type III diseases in some siblings and possibly also in single subjects.

Manners and Wright[35] have recently divided limit dextrinosis into three subgroups based on the presumed involvement of a trans-α-glucosylase. We have found no evidence of the action of such an enzyme in glycogen degradation; hence, any subdivision of limit dextrinosis seems unwarranted.

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Phenylketonuria

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THE observation by Fölling[1] that certain mentally defective subjects excreted phenylpyruvic acid in the urine represents the first demonstration of a specific chemical defect in a mental disorder. Although there have since appeared other substantiated examples of such chemical correlations with mental disease, phenylketonuria remains the best studied example of them all. Since Fölling's original observation, we have learned a great deal about the genetic nature of this disorder, the readily observable enzymatic lesion and even about effective therapy. However, the most interesting problem of all, the one concerning the reasons for the mental derangement in this disease, is still unsolved. Solution of this problem may well pave the way for future advances in the chemical pathology of the central nervous system.

Phenylketonuria is transmitted as a recessive characteristic from a single autosomal gene. The heterozygous parents, who apparently are normal, give rise to a certain percentage of homozygous phenylketonuric offspring. The incidence of phenylketonuria in the general population has been estimated at between one in 25,000 to one in 50,000.[2] It may be estimated, therefore, that one person in 100 may be a heterozygous carrier of this disease.

Advances in methodology have made it possible to detect and measure many chemical changes associated with this disorder, and we know that, in addition to the excretion of phenylpyruvic acid in the urine, there is a marked elevation in phenylalanine blood levels. (Normal subjects, 1 mg. per 100 ml.; untreated subjects with phenylketonuria, 20 to 60 mg. per 100 ml.). The large amounts of phenylalanine represent an inability to metabolize this amino acid, and it is now recognized that this is due to a block in the major route of phenylalanine metabolism, hydroxylation to tyrosine.

Phenylalanine hydroxylation requires two protein catalysts, one is present only in liver and the other is more generally distributed. Additional requirements are reduced pyridine nucleotide, and a pteridine-like cofactor (Fig. 1). Studies in several laboratories have pinpointed the lesion as being a marked deficiency in the liver enzyme, the other enzyme and the cofactors being present in normal amounts in the liver of phenylketonuric subjects. If we accept this one enzyme deficiency as the only genetically linked disturbance, then it must be concluded that phenylketonuria is primarily a hepatic disorder and that the mental retardation is a secondary effect. I would like to discuss some possible mechanisms of such a secondary effect.

PHENYLALANINE METABOLISM

In the absence of phenylalanine hydroxylase, phenylalanine metabolism is markedly diminished. Large amounts of the amino acid persist in the tissues for sufficiently long periods of time so as to yield quantities of products which are normally formed and excreted only in trace amounts (Fig. 2). Thus, transamination of phenylpyruvic acid becomes a major metabolic pathway. O-Hydroxyphenylacetic acid, which is normally excreted in microgram amounts, is excreted to the extent of several hundred milligrams per day. Many other acidic and phenolic products have been found in the urine and blood of phenylketonuric subjects but none possess pharmacologic ac-

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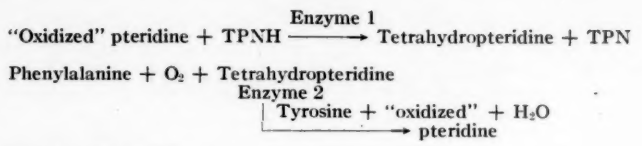

Enzyme 1—Many Tissues
Enzyme 2—Liver only (Absent in Phenylketonuria)

FIG. 1. Enzyme and cofactor requirements for hydroxylation of phenylalanine.

tivity which can be associated with a centrally active agent. Recently Jepson et al.[3] reported that traces of phenylethylamine are normally excreted in the urine. Following administration of monoamine oxidase inhibitors, phenylketonuric subjects excreted more than 3 mg. of phenylethylamine per day, whereas normal subjects excreted less than 0.05 mg. per day. Since even with monoamine oxidase inhibitors intravenously administered phenylethylamine was poorly recovered in the urine, it was estimated that in phenylketonuric subjects as much as 50 mg. of this amine may be synthesized per day.

Unlike the phenolic acids, phenylethylamine is a pharmacologically active substance which possesses properties like amphetamine, although of weaker activity. It is now known that phenylethylamine is formed by decarboxylation of phenylalanine in mammalian tissues. It is of further interest that the enzyme, aromatic L amino acid decarboxylase, is present in many organs. It is also present in brain in which it is localized in the brain stem areas.[4] Furthermore, the affinity of phenylalanine for the decarboxylase is low so that saturating levels are not reached. In the presence of increased amounts of phenylalanine, the rate of decarboxylation is increased. Thus, in the brains of phenylketonuric subjects it is quite probable that phenylethylamine production takes place at a rate twenty to sixty times normal, since it is fairly certain that brain levels are comparable to blood and spinal fluid levels. Phenylethylamine may very well be the "*neurotoxic*" substance suggested by Armstrong[5] and by others. It may be that the presence of such a pharmacologically active substance during postnatal development of the brain may either interfere with normal learning (imprinting), or it may have more direct effects on brain chemistry. Such effects could very well be irreversible.

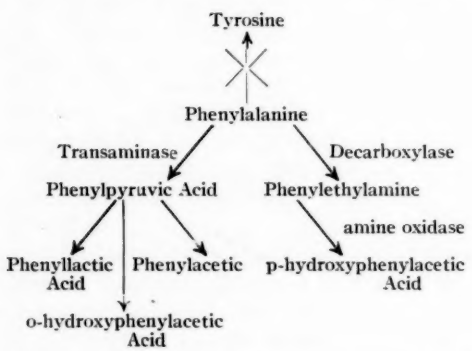

FIG. 2. Alternate pathways for phenylalanine metabolism.

Another suggested mechanism for the central defect comes from the reports of Pare, Sandler and Stacey[6] on 5-hydroxyindole metabolism. They have shown that both plasma serotonin and urinary 5-hydroxylindoleacetic acid levels are considerably lower in phenylketonuric subjects as compared to other mentally defective subjects. Sandler and Stacey discuss the possibilities of intereference with tryptophan hydroxylation or 5-hydroxytryptophan decarboxylation by phenylalanine or its metabolites. An alternate explanation of their findings is interference with transport mechanisms for serotonin and its metabolites in kidney and blood platelets. Of course, we still know little about the function of serotonin in brain. The large amounts of phenylalanine and its metabolites appear to influence many metabolic pathways including those relating to tryptophan, catecholamines and γ-aminobutyric acid. Any one of these may be of significance in the brain disturbance.

TABLE I

Effects of Amino Acid Antagonists on Increase in Brain Levels of L-Tyrosine After its Administration

Amino Acid	Increase in Brain Level (μg./gm.)		
	30 Minutes	60 Minutes	120 Minutes
Control	44 ± 2	61 ± 2	62 ± 3
L-Tryptophan	9, 12, 5, 8	24, 26, 15, 23	60, 33
β-Fluorophenyl-DL-alanine	11, 4	25, 13	28
L-Leucine	2, 1	7, 7	21, 30

That phenylalanine, itself, can interfere with other enzymes was definitely shown by the studies of Dancis et al.[7] on the enzyme tyrosinase. They showed that this enzyme system, which is involved in melanin formation, is markedly inhibited by concentrations of phenylalanine which are found in tissues of phenylketonuric subjects. They concluded that the blond hair and light coloring were the result of significant interference with pigment formation by the high levels of phenylalanine in the tissues.

Another type of interference which could specifically influence brain nutrition is one with aromatic amino acid transport. It has been shown by Chirigos et al.[8] that the uptake of tyrosine by brain, *in vivo*, depends on a catalytic mechanism. The following evidence is given (1) uptake is rapid; (2) the uptake is stereospecific; L-tyrosine is taken up much more rapidly and to a much greater extent than the D-isomer; (3) compounds structurally related to tyrosine, with similar solubility charactertisics, are not taken up to an appreciable extent; and (4) uptake is markedly diminished in the presence of certain other amino acids, L-tryptophan and fluorophenylalamine (Table I), indicating competition for the uptake mechanism. Phenylalanine could not be studied as a competitor since in normal animals it is rapidly converted to tyrosine. Neither structural specificity nor competitive inhibition by other aromatic amino acids were observed in tyrosine uptake by other tissues such as muscle *in vivo* or in *in vitro* and even brain slices do not possess the same characteristics as the *in vivo* organ.

TABLE II

Phenylalanine Hydroxylase Activity in Liver of Premature Human Infant

Preparation	Additions	Tyrosine Formed (μM)
Soluble fraction	...	0.0
Soluble fraction	Rat liver Enzyme 2	0.73
Soluble fraction	Rat liver Enzyme 1	0.0
...	Enzyme 1 + 2	0.98

NOTE: Enzyme 2 = hydroxylase. Enzyme 1 = pteridine reductase.

It would appear that the catalyzed uptake of L-tyrosine (and other normal amino acids) by brain occurs at the same site as the so-called "blood-brain barrier." The presence of a catalytic mechanism for uptake of charged water-soluble nutrients into brain differentiates it from other organs and indicates that brain uptake should be susceptible to competitive inhibitors and other factors which can influence catalytic mechanisms. For this reason large amounts of one aromatic amino acid can competitively inhibit the uptake of another into the brain. Such a mechanism could explain a central action of the huge amounts of circulating phenylalanine in phenylketonuric subjects. A diminution in uptake of essential amino acids, would, of course, have many consequences.

NUTRITIONAL ASPECTS

Although the reason for the central defect is not certain, it is now possible to remove all chemical manifestations of this disorder by lowering the dietary intake of phenylalanine sufficiently. If such dietary restriction is imposed from birth even the central aspects of this disorder can be averted.[9] Studies of Kenney and Kretchmer[10] indicate that there is no distinction between normal and phenylketonuric subjects at the fetal stage, neither containing the enzyme, phenylalanine hydroxylase (Table II). No damage should therefore occur *in utero*.

Following birth, plasma levels of phenylalanine do not rise until protein feeding is instituted. Commercially prepared low phenylalanine diets started at, or shortly after, birth appear to have a definite effect in preventing mental deficiency. However, for proper use of the diet early diagnosis is required. In Cincinnatti newborn children are sent home with filter paper patches inserted in the diapers. The patches are sent back to the laboratory for phenylpyruvic acid testing. Newborn children in families with a history of phenylketonuria should have phenylalanine blood levels assayed at frequent intervals until it can be ascertained whether they are normal or not. If high levels are found, the low phenylalanine diet should be instituted.

Detection of the heterozygous carriers of phenylketonuria appears to be a distinct possibility. Hsia et al.[11] and Knox and Messinger[12] have reported that in parents and normal siblings of phenylketonuric subjects an orally administered dose of L-phenylalanine cannot be metabolized as rapidly as in normal subjects. An oral phenylalanine tolerance test appears to distinguish heterozygotes from noncarriers. The ratio of phenylalanine to tyrosine in blood[13] and the amount of orthohydroxyphenylacetic acid in urine[14] following the oral administration of phenylalanine also appear to distinguish the heterozygote group. However, although these tests appear to be able to distinguish one group from another, they are not yet able to detect the phenylketonuric trait in a given subject because of the considerable overlap between the normal and heterozygous groups. Perhaps with more refined technics detection in given subjects may become possible.

One of the factors which has limited experimentation in this field has been the lack of an experimental animal. However, it now appears that Waisman and associates[15] have been able to produce all the chemical manifestations of phenylketonuria in monkeys by feeding large amounts of phenylalanine in the diet from birth. Psychologic testing indicates that these monkeys grow up mentally retarded. Such a test animal should prove most important in corroborating our present concepts of this disorder and in exploring the mechanism of the central defect.

It is of interest that a comparable disorder involving the leucines and valine has been discovered.[16] The term "maple-syrup" urine disease has been used because of the odor of some of the excretory products. As in phenylketonuria, the amino acid levels are markedly elevated, keto acids are excreted in the urine and central disturbance is marked. Genetically, the two are also similar. It will be of interest to compare these two disorders in greater detail as more cases become available.

SUMMARY

It is apparent that we already know more about phenylketonuria from the genetic, biochemical and therapeutic standpoint than we do about other comparable disorders. However, recent advances indicate that we may also be able to investigate the causes of the central disturbance.

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Wilson's Disease

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WHY does a disease as rare as Wilson's disease attract sufficient interest to warrant its inclusion in this program? I believe the chief reason is that despite its complexity and clinical variation there is a growing conviction that the disease must be caused by a fairly simple primary defect and that many, if not all, the clinical vagaries will be explicable when the nature of the primary defect is recognized.

If I can summarize, and necessarily oversimplify the clinical aspects of the disease, it will perhaps enable the biochemistry and genetics to be put in perspective. In 1911 Kinnier Wilson[1] described a disease which he called "progressive lenticular degeneration, a familial nervous disease associated with cirrhosis of the liver." The disease is extremely variable; in some instances after an early onset, rigidity and muscle spasms, dysphagia and dysarthria are associated with a progressively downhill course and death ensues within a few months. In others, a slow insidious tremor in the early twenties is the only sign of the affliction. Other patients show a gradual deterioration over the years which may still leave the patient ambulant and enjoying life twenty five years after the onset. Sometimes the disease is heralded by hematemesis or ascites and cirrhosis of the liver. Progressive liver failure may dominate the clinical picture. Of great diagnostic importance is the fact that Kayser-Fleischer rings are nearly always present once the disease is recognizable clinically. Epileptic fits, broken bones, arthritis, azure lunulae and pigmentation are among the strange potpourri of symptoms which are present in the disease. Is it reasonable that these widespread and varying symptoms could stem from a single biochemical lesion?

Wilson postulated that the familial nature of the disease was due to nongenetic causes and for a number of years the mode of inheritance was in doubt. A recessive mode of inheritance was suggested[2] but the data in the literature were hard to disentangle due to insufficient information on the numbers of unaffected sibs and relatedness of the parents. The occasional occurrence of the disease in two generations was incorrectly regarded as strong evidence against a recessive inheritance. Collection of pedigrees in recent years and a greater awareness of genetic disorders has resulted in the disease being established as inherited in an autosomal recessive fashion. A high consanguinity rate in the parents, the best evidence for a rare recessive, could be demonstrated,[3] and genetic calculations supported this assumption.

According to current theory, a mutation can be considered an event which alters the base sequence in DNA and as a direct consequence alters the synthesis of a particular protein. This alteration can so upset the program that no recognizable protein can be synthesized or it may only change the message slightly so that a different protein is made. This concept is somewhat analogous to Muller's system of amorphs (or hypomorphs) and neomorphs in new guise. Once it is decided that the disease is due to a single gene in double dose, the ultimate question is what enzyme or protein is disturbed by the mutation. What is the primary gene product of the normal allele at the Wilson's disease locus and is it the same as the product in persons suffering from the disease? Despite the apparent complexity of the disease, its recognition

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as an autosomal recessive has enabled the formulation of precise questions although precise answers have not yet been forthcoming. It may be useful to attempt to appraise our present knowledge of Wilson's disease in this light.

ROLE OF COPPER

Recognition that copper accumulation was an integral part of Wilson's disease was the undoubted beginning of the resurgence of interest in Wilson's disease during the last two decades. These observations were made primarily by clinicians and pathologists who painstakingly examined the tissues of patients who died from the disease. An increased copper content of the body was recognized and it was also shown that the urinary excretion of copper was elevated compared with several neurologic conditions. At about this time, Holmberg[4,5] in Sweden was shown a sample of pig serum which exhibited a distinct blue color. In association with Laurell, isolation and characterization of this blue protein was carried out with great success both in pig serum, and later in normal human serum. The protein proved to be an α_2 protein with a molecular weight of about 151,000 and to have 8 atoms of copper per molecule. The protein behaved like a laccase having oxidase activity towards a variety of substrates but particularly toward paraphenylene diamine.[4,5] It is noteworthy, however, that compared with other oxidases, the activity is very low and whether or not the protein acts as an oxidase *in vivo* is still much in debate. Scheinberg and his colleagues[6–8] have shown that not all the 8 atoms of copper are equally firmly bound. Half can be removed with relative ease and under the right circumstances the copper could be put back on the molecule and activity almost completely restored.[6] An attractive hypothesis was proposed by these workers[7] who suggested that ceruloplasmin might act as a transport protein for copper *in vivo* similar to transferrin for iron transport. However, more recent observations by the same authors[8] provide no evidence that the exchange of copper atoms occurs *in vivo* and other functions for this unique copper protein must be sought.

CERULOPLASMIN

With the recognition that there was excess copper in the body of patients with Wilson's disease, it was a short step to determine the serum copper and ceruloplasmin level in this condition. Surprisingly, despite the excess of copper in the body, the serum copper and ceruloplasmin levels were markedly depressed.[9] Fortunately the disease was becoming of interest to a variety of workers in the field and confirmatory reports of depressed copper and ceruloplasmin quickly appeared.[10,11] A simple hypothesis promptly emerged that perhaps the disease was due to an inability to synthesize the protein ceruloplasmin. If this protein regulated the absorption and/or excretion of copper, a decreased level would cause a net increase in the copper content of the body. With the passage of time this hypothesis has been increasingly hard to accept in its simplest form.

There is no doubt that accumulation of copper in the liver can occur very early. In an asymptomatic three and a half year old sib of a patient with Wilson's disease, Scheinberg[12] has demonstrated a grossly increased copper content of the liver which, apart from the presence of large glycogen nuclei in the liver, did not reveal any gross histologic changes. If this patient is homozygous for the Wilson's disease gene, it has been demonstrated that copper accumulation precedes the development of the cirrhosis. Serial biopsies will be of great interest. The demonstration of large quantities of copper in all parts of the brain correlates with the widespread neurologic symptoms: the disease is a good deal more than pure lenticular degeneration. Copper can be shown to accumulate in the renal tubules and that the nature and extent of the renal lesion is related to the duration of the disease. At first the brunt of the damage falls on the proximal renal tubule and glycosuria, phosphaturia, aminoaciduria and uricosuria ensue. These substances are presumably reabsorbed through mechanisms which are relatively easily poisoned by copper. Later distal tubular functions fail and the circulatory dynamics of the kidney—the glomerular filtration rate and the effective renal plasma flow—become disturbed

The renal lesion has attracted considerable interest since this disturbance of metabolism may be of primary importance. Beta amino isobutyricaciduria, cystinuria and glycinuria are all inherited conditions in which the primary effect of the gene is a disturbance of amino acid metabolism transport. The belief that Wilson's disease might represent a form of inherited aminoaciduria was strengthened by the finding that increased amino acid excretion occurred in some relatives. However, this was not revealed in the majority of relatives of patients with Wilson's disease, and most workers are inclined to regard the amino acid disturbances as secondary phenomena.

There is no doubt that in patients with Wilson's disease there is an increase in the total body copper, although whether or not this is due to increased absorption or decreased excretion is uncertain. Recent evidence of Gitlin and his co-workers[13] suggests that more attention should be paid to the possibility of decreased copper excretion in this disease. The copper cannot be incorporated into ceruloplasmin, resulting in an increased quantity of copper perfusing the tissues loosely bound to serum albumin. Whenever there are substances in the tissues, polypeptides or proteins, which have an affinity for copper greater than albumin, the copper will be deposited and disease will result.

A number of observations at variance with this hypothesis should be mentioned. There is no correlation between the depression of the concentration of ceruloplasmin and the severity of the symptoms. Indeed there are a number of patients with unequivocal Wilson's disease who show normal levels of ceruloplasmin. Moreover, a depression of ceruloplasmin can occur in conditions other than Wilson's disease, although admittedly the duration of the depression is usually considerably shorter. Such conditions include nephrosis, generalized hypoproteinemia and perhaps sprue. Some heterozygotes, as will be discussed, may also have severely depressed ceruloplasmin levels without overt symptoms of disease.

If we wish to continue to regard ceruloplasmin deficiency as a primary disturbance, we are forced to the hypothesis that the disease may be present in at least two allelic forms. In the first and more common, the ceruloplasmin is synthesized at a decreased rate; in the second, the rate of production is almost the same but the product differs. The first corresponds to Muller's hypomorph and a nonsense mutation in the code; the second relates to a mutational event which results in the formation of a neomorph (missense mutation). However, there is no proof, of which I am aware, that the decreased ceruloplasmin in patients with Wilson's disease is structurally normal. Additional studies are urgently needed.

CLINICAL STUDIES

Our series of cases collected in New York were analyzed genetically. The patients could be divided into two main groups: those who came from Eastern Europe and Poland and those who came from Italy. Clearly the disease has occurred all over the world, but the concentration of cases in these two populations appeared worth investigating in an effort to gain further insight into the possibility of heterogeneity. The advantages of looking at genetic data in this way should be emphasized for it may give evidence of the existence of modifying genes present in certain populations which may or may not have important selective advantages. The two groups appear to be slightly different statistically, particularly if several aspects are combined. By and large it appears that in those from Eastern Europe the age of onset is slightly later, the disease milder and the disturbance in Darwinian fitness less than in those from Sicily and Southern Italy. Biochemically there is little to differentiate them. The possibility that the ceruloplasmin may, on the average, be higher in those from Eastern Europe, is not yet established. Thus, there is suggestive genetic and chemical evidence that in some patients the disease may be caused by a different allele.[2] However environmental factors may play a role in the differences between the two groups.

It would be of great importance to know the frequency of the gene in the population. Unfortunately all calculations are very approxi-

mate. Moreover the sample is small. Theoretically the gene frequency might differ in those from Eastern Europe, from Italy and in the general population. Methods for calculating the gene frequency are well established. One method utilizes the relationship between gene frequency and marriages between cousins. However, the incidence of marriages between first cousins in the population from which the sample is drawn must be known. The calculation can be performed with first cousin, second cousin and unrelated parents and q should be the same in the three calculations. However, between groups the value for q might vary widely.

Calculation of the mutation rate is virtually impossible with a rare recessive. The formula of Morton and Chung,[14] in this instance, is of dubious value. The formula is usually expressed $M = q(\alpha^1 + q + h) S$ where α_1 is the long-term inbreeding coefficient and h is the selective coefficient against heterozygotes. α_1 is about 0.006 and h is presumably 0. The formula assumes an equilibrium between selection and mutation. The important point, however, is that we do not have an accurate value for q or S, and it seems unlikely that in the present state of ignorance it is worth calculating the mutation rate for the Wilson's disease gene. A very small advantage of the heterozygote is needed to balance even a lethal homozygote. The advantage has to be roughly proportional to the gene frequency.) Thus, although there may be a heterozygous advantage in Wilson's disease, it will not be detectable under ordinary circumstances.

HETEROZYGOTES

Let us return now to the question of the detection of carriers of the Wilson's disease gene. Only a decade ago, apart from the blood groups, there was no way of detecting heterozygotes with confidence. In the last few years it has become very important to devise methods for detecting the heterozygote. This is not primarily for so-called eugenic reasons but to enable us to understand more about the possible origins and spread of the disease. As a general rule, the ease with which heterozygotes can be detected will depend on how close one is to the primary gene product. Theoretically, if one were in a position to catch the substance as it comes off the ribosomes then in principal the two products of the heterozygote could be detected. The further away from the gene product, the more likely will genetic modifiers and environmental factors influence the detection of the heterozygote. In Wilson's disease a significant proportion of the parents show a decreased quantity of ceruloplasmin. However, the quantity is by no means invariably depressed. Some recent observations from Scheinberg's group have revealed that the use of radioactive copper will disclose a larger percentage of carriers than the determination of ceruloplasmin alone.[15] However, even with this method there is considerable overlap between the groups. This is not unexpected and moreover, the degree of overlap is an important genetic parameter to define. One can be certain that measurement of the ceruloplasmin concentration in the serum ultimately will not prove to be the best way to detect the presence of the Wilson's disease allele.

How can we explain the patients with normal ceruloplasmin? It seemed possible that although the ceruloplasmin was normal in quantity perhaps it was abnormal in quality. In other terms a missense mutation has resulted in the formation of an abnormal ceruloplasmin with different properties. When Broman[16] had shown that ceruloplasmin could be fractionated into two peaks on hydroxyapatite columns, it became of interest to separate the serum of patients with Wilson's disease under identical conditions. It was found that patients with normal and low values of ceruloplasmin had similar ratios of activity in the two peaks, although of course each peak was greatly decreased in those with low levels. In one experiment the ceruloplasmin level was raised by the administration of estrogens and the ratio of activity in the two peaks remained the same at widely differing ceruloplasmin values. This evidence, as far as it goes, shows no difference in the ceruloplasmin. Clearly more work is necessary for it is by no means conclusively proved that the reported heterogeneity of ceruloplasmin is genuine heterogeneity, and not

artifacts produced by the experimental conditions.[17]

SUMMARY

Whatever the cause of Wilson's disease there is one clear cut biochemical abnormality: an increased total body copper. It seems therapeutically plausible to remove the excess copper as quickly and completely as possible. This can be achieved by oral administration of penicillamine. With judicious but humane advice concerning the avoidance of foods containing a high copper content and the regular administration of penicillamine, the copper content of the body returns toward normal and in some cases is associated with undoubted clinical improvement. Replacement of a low ceruloplasmin level either by estrogen administration or by infusion of ceruloplasmin seems of little value. The regular infusion of apoceruloplasmin, to prevent absorption of copper and penicillamine to eliminate the copper already present, would be logical therapy for this particular inborn error of metabolism and already the therapeutic nihilism epitomized in the statement that inherited diseases are cured only by the grave is happily mistaken.

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Sensitivity to Hemolytic Drugs

An Inborn Error of Metabolism of the Red Cell

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THOSE charged with the care of the sick have often been dismayed to find that a drug innocuous to many, may produce catastrophic results in a few. Until recently, attempts to explain unusual reactions to drug therapy have ended in failure because untoward drug reactions usually occur sporadically and are often so dangerous that re-administration of the drug to the person manifesting the idiosyncrasy under study is out of the question. In 1953, my colleagues and I were presented with the unique opportunity to study in detail one such drug-sensitivity reaction, the hemolytic effect of the 8-amino-quinoline antimalarial compound, primaquine. These studies not only helped clarify the mechanism of sensitivity to this drug, but also led to the demonstration that a hereditary biochemical lesion of erythrocytes was responsible for many other drug-induced hemolytic anemias and for favism.[1]

The clinical manifestations of drug-induced hemolysis are well known.[2] Between one and three days after the patient begins to take the drug, the hemoglobin level of the blood falls, the urine darkens and the scleras become icteric. In severe cases, pain in the abdomen and the back may also occur. Usually, the patient recovers, but occasionally he succumbs, either to the severe anemia or to the nephropathy which follows massive blood destruction. In the case of favism, onset of hemolysis may be much more sudden, occurring within a very short period of time following the ingestion of fava beans.[3]

PRIMAQUINE SENSITIVITY

When the antimalarial compound, primaquine, was given to volunteer subjects, acute hemolytic anemia occurred in a few recipients. Others showed no evidence of hemolysis.[4] The now classic studies of Dern and his associates[5] demonstrated quite clearly that sensitivity to hemolysis was due to an intrinsic abnormality of the erythrocyte. When red cells from a drug-sensitive subject were labeled with Cr^{51} and given as a transfusion to nonsensitive recipients, the red cell survival time became remarkably shortened when the drug was administered. Conversely, red cells from a nonsensitive subject survived normally in the circulation of a sensitive person, even when the drug was administered to him and hemolysis resulted, as evidenced by a fall in the hematocrit.

Early in the course of studies of hemolytic anemia induced by primaquine therapy, a peculiar phenomenon was observed: when administration of the drug to a sensitive subject was continued, evidence of hemolysis abated spontaneously, hemoglobin levels returned to normal and the patient remained perfectly well, even when the dose of primaquine, which originally had elicited hemolysis, was doubled. Inspection of some of our survival curves suggested that the sensitivity to hemolysis might be a function of red cell age. Therefore, a primaquine-sensitive subject was given Fe^{59} so that a portion of his red cell population

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of narrow age range would be labeled.[6] Administration of the drug to this patient produced no hemolysis of labeled cells when the cells were aged from eight to twenty four days; however, when the cells were allowed to age longer in the circulation, a second course of drug administration (while the cells were sixty-three to seventy-six days of age) produced brisk destruction of labeled erythrocytes.

Earlier studies had demonstrated that persons who were sensitive to primaquine were also sensitive to other 8-amino quinoline antimalarials.[4] The Cr^{51}-labeling technic afforded us an opportunity to study the spectrum of drug sensitivity with a minimum of risks to the patient. Transfusion of red cells from sensitive and nonsensitive subjects were given to nonsensitive recipients and the rate of disappearance of red cells from the circulation was studied when the various drugs were given. In this way, it was determined that primaquine-sensitive red cells were also uniquely sensitive to the hemolytic action of a variety of other drugs, including sulfanilamide, acetanilid, phenylhydrazine, phenacitin and many others.[7] More recently, it has been found that nitrofurantoin,[8] and even aspirin when given in large doses,[9] may cause selective destruction of sensitive erythrocytes. It has also been learned that the red cells of subjects with sensitivity to the hemolytic effect of the fava bean are invariably primaquine-sensitive. The reverse, however, is not true. Primaquine-sensitive subjects frequently do not manifest the symptoms of hemolytic anemia when they are exposed to fava beans.[10]

It was clear, then, that persons who were primaquine-sensitive had red cells which were different from those of nonsensitive subjects. They were uniquely sensitive *in vivo* to the hemolytic effect of a variety of drugs. It was also clear that only the members of the red cell population of such persons were destroyed when the drug was given.

BIOCHEMICAL CONSIDERATIONS

But how did these cells differ from normal cells? When examined by classic hematologic technics, they appeared to be perfectly normal. Their morphology was normal on a wet-mount or unstained smear. No coating antibodies could be demonstrated. These cells were not uniquely sensitive to lysis *in vitro* by primaquine.[11] However, it was possible to show that the pattern of Heinz body formation in sensitive red cells is different from that of nonsensitive cells, when incubated under carefully standardized conditions with acetylphenylhydrazine.[12] Sensitive cells formed many small Heinz bodies in each cell, but sensitive cells, in general, formed only a few large Heinz bodies.

A number of biochemical investigations of primaquine-sensitive and nonsensitive red cells proved fruitless, but it was possible to demonstrate that the level of reduced glutathione (GSH) was rather consistently decreased in sensitive cells. However, we were not satisfied that glutathione deficiency was the primary defect of drug-sensitive cells, for the difference between normal and sensitive cells was small. There was also some overlap between sensitive and nonsensitive subjects when sufficient number of volunteer subjects were investigated, especially if only single determinations, rather than the average of several days determinations, were considered.

It was discovered that when drug-sensitive red cells were incubated with a reducing agent such as acetylphenylhydrazine, there was rapid destruction of red cell GSH; under identical conditions no GSH destruction occurred in nonsensitive erythrocytes.[13] I have called this test the glutathione stability test. It is a convenient way of demonstrating *in vitro* the presence or absence of drug sensitivity. It has also been of some help in further elucidating the basic metabolic disorder of these erythrocytes.

When GSH is destroyed in sensitive red cells under the influence of acetylphenylhydrazine, much of it is converted to the oxidized form of glutathione. Some is probably also bound to hemoglobin.[14] If we consider for a moment the mechanism by which GSH is normally maintained[15] in the reduced form in erythrocytes, we see that it is necessary for

TPNH to be present as a hydrogen donor in the reaction

$$GSSG^{*} + TPNH + H^{+} \rightleftarrows 2\,GSH + TPN^{+}$$

The red blood cell has two main pathways for the metabolism of glucose; the anaerobic, glycolytic pathway, and the direct oxidative pathway, or hexose-monophosphate shunt. It is in the latter pathway that TPN is reduced to TPNH. Investigations by Carson and others[16] of the three enzymatic steps, inhibition of which would result in failure to reduce oxidized glutathione properly, namely, glucose-6-phosphate dehydrogenase, phosphogluconic dehydrogenase or glutathione reductase, demonstrated that drug-sensitive red cells are deficient in glucose-6-phosphate dehydrogenase (g-6-pd). It has seemed likely that this is the primary defect of primaquine-sensitive red cells.

It is possible, however, and indeed there is now evidence to suggest that, at least in Sefardic Jews, the enzyme itself may be normal but that an activator is missing. If hemolysate from g-6-pd-deficient Sefardic Jews is incubated with normal stroma, there is marked activation of the enzyme.[17] Studies are under way in our laboratory to determine whether the same effect can be produced with the red cells of sensitive Negro subjects. We should like to determine whether stroma from Negro subjects with this red cell defect can reactivate enzyme in hemolysate from Sefardic Jews, since, as will be pointed out, there seem to be basic differences in this disorder between Caucasians and Negroes. Thus, it is possible that there may be mutual correction of the defect when stroma and hemolysate from Caucasian and Negro subjects are mixed.

A POSSIBLE EXPLANATION

Let us speculate briefly on how a deficiency of the GSH-reducing system might lead to destruction of red cells when a drug is administered. When a drug is given to a nonsensitive subject, we postulate that a reversible change in the hemoglobin molecule within red cells occurs. In the presence of GSH, this damage is repaired. In the process, GSH is oxidized to GSSG, but the latter compound is quickly restored to the reduced form, GSH, which now stands ready to protect the hemoglobin from assault by drugs. In the drug-sensitive cell, when GSH is oxidized in repairing the damage to the hemoglobin molecule, it is not again reduced to GSH because of the deficiency of glucose-6-phosphate dehydrogenase. Further, there is evidence to suggest that GSH synthesis is defective in sensitive cells.[18] As repeated assaults are made by the drug on the hemoglobin molecule, the damage becomes irreversible. Heinz bodies form within the red cells, and the cell is selected for destruction by the reticuloendothelial system in an, as yet, unknown manner. I must emphasize that this is only a hypothesis and that the exact mechanism of action of drugs in producing hemolysis of sensitive cells is as yet unknown. However, I believe that this hypothesis is a plausible one and is based on many findings.[14,19]

GENETIC ASPECTS

It has been established clearly that primaquine sensitivity is genetically transmitted. The mode of transmission has been elucidated by the studies of Childs et al.[20] Szeinberg and his associates,[10] and by others.[21] The defect is a sex-linked one and is transmitted presumably on the X chromosome, from the mother to her daughters or to her sons, but by the father only to his daughters. The penetrance of the gene for primaquine sensitivity is variable in the female. In the affected male, the full-blown typical enzyme deficiency and drug sensitivity is present. However, in the female, having one chromosome carrying the defective gene and one carrying the normal gene, the expression may vary from full expression to no detectable expression at all. It is presumed that the homozygous female invariably has the severe defect.

The distribution of this defect is now known to be worldwide.[21] It is particularly common in areas in which the incidence of malaria is high. Motulsky[22] has suggested that this defect may have survived in these populations by conferring an increased resistance to malaria. The severity appears to differ in Caucasian, as compared with Negro populations who are

* Oxidized glutathione.

affected.[23] It may be that two separate mutations have arisen in these populations, both with the same final effect but conferring varying degrees of enzyme deficiency. In addition, a biochemically similar, but perhaps more severe, defect has been observed among certain subjects with nonspherocytic congenital hemolytic anemia.[24] Thus, a third mutation appears to result in so severe a defect in the enzymatic activity of erythrocytes that clinically apparent shortening of red cell survival occurs.

Since g-6-pd deficiency is genetically determined one might suspect that severe depletion of this enzyme would be found in all somatic cells. While it has been possible to demonstrate deficiency of this enzyme in various cell types of Caucasian subjects,[25,26] this has been more difficult to achieve in Negro subjects with this abnormality.[27] It is possible that the enzymatic deficiency is particularly severe in erythrocytes because they are nonnucleated and, therefore, do not have the capacity to synthesize protein once they are launched from the marrow to complete their 120 day life span in the circulation. Indeed, it has been demonstrated[28] that the activity of g-6-pd diminishes in normal erythrocytes as they age and virtually disappears from the erythrocytes of those with g-6-pd-deficiency. This probably explains the greater susceptibility of older red cells to hemolysis.

Much remains to be learned about the exact nature of this defect and the way in which it renders affected persons sensitive to the hemolytic effect of drugs. Much, however, has already been learned and the road of exploration has been exciting.

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Clinical Reports

Effect of Food on the Absorption of Vitamin B_{12}

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THE vitamin B_{12} urinary excretion test[1] has been used frequently for the measurement of absorption of vitamin B_{12}. The procedure of this test involves the oral administration of a test dose of the labeled vitamin, followed by the intramuscular injection of a large dose of unlabeled vitamin in order to "flush out" the absorbed radioactive vitamin. Measurement of radioactivity in urine specimens collected for twenty-four hours, or longer, is used as an index of absorption. Such data yield arbitrary but reproducible values if the test conditions are standardized and if the physiologic state of the volunteer subjects is essentially uniform. Although this test has been used by a number of investigators, the effects of slight variation of the test conditions, such as prefeeding shortly prior to the administration of radioactive vitamin B_{12}, have not been carefully studied. The purpose of this report is to study the influence of prefeeding on the urinary excretion of radioactivity from labeled vitamin B_{12} in man and on the deposition of radioactivity in selected tissues in rats. Inferences about absorption will be made from the tracer studies.

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MATERIALS AND METHODS

The test procedure used is essentially that described in a previous paper.[2] Three experiments with human subjects were carried out. In one, twenty male volunteer subjects (inmates of a state penal institution) twenty-four to forty years of age, clinically healthy and with substantially uniform dietary histories over the recent past, were assigned at random to one of two groups. One group (ten subjects) was offered breakfast consisting of liberal amounts of dry cereal with milk, pancakes and bacon; the other (ten subjects) was fasted from 4 P.M. of the previous day.

Within thirty minutes after breakfast subjects in each of the two groups, were given capsules containg 50 μg. of labeled vitamin B_{12}. Two hours later, each subject was given an intramuscular injection of 1,000 μg. of unlabeled vitamin B_{12} and the urinary output of each was collected for the next twenty-four hours. Half of each sample was evaporated on the steambath to less than 50 ml. and transferred quantitatively to a brown bottle calibrated at 50 ml. Radioactivity was measured in a gamma scintillation counter.

In the second study, twenty-three subjects were divided into two groups. Subjects in group A (twelve subjects) were fasted; those in group B (eleven subjects) were given breakfast as described. Two hours later all subjects were given 10 ml. of aqueous solution containing 50 μg. labeled vitamin B_{12}. Quantitative ingestion of the dose was assured by repeated rinsing of the containers with 10 ml. of water. Each subject drank four such washings. In the third study, two groups of five subjects were used.

TABLE I
Studies in Man
(Urinary Excretion of Radioactivity*)

Study	A (with breakfast)	B (without breakfast)	A:B
1	1.60 ± 0.072(10)†	0.82 ± 0.054(10)	1.94
2	2.72 ± 0.24(11)	1.39 ± 0.07(12)	1.96
3	2.00 ± 0.20(5)	1.22 ± 0.20(5)	1.64

* Values are expressed in per cent of the administered dose of 50 μg./$Co^{60}B_{12}$.
† Numbers in parentheses denote number of subjects (human) used.

A third experiment on man was conducted differing from the first two in that the breakfast of the prefed group (five subjects) consisted of a large portion of corn flakes, milk and sugar, *ad libitum* (Table I). Immediately after breakfast, they were given 50 μg. radioactive vitamin B_{12} orally. They excreted 2.00 ± 0.2 per cent of the ingested radiovitamin in the twenty-four-hour urine. For comparison, the fasted group was fed the same amount of vitamin B_{12} and the five subjects excreted 1.22 ± 0.20 per cent only. The 66 per cent increase in excretion is again statistically significant ($P < 0.025$).

To study the effect of prefeeding on the absorption of vitamin B_{12} in rats, male adults were fasted overnight. On the following morning, half of them were offered 5 gm. portions of a casein diet. Feed cups were withdrawn half an hour later. Any unconsumed food was then weighed. The feed intake varied from 1 to 5 gm. per rat. Precisely thirty minutes later, 50 mμg. of radioactive vitamin B_{12} was given by stomach tube to both fasted and fed animals. Feces were collected for four days, after which the animals were sacrificed and their livers and kidneys removed. The feces and organs were solubilized with concentrated H_2SO_4 or KOH solution for radioactivity measurement.

RESULTS

Table I summarizes the findings of our first experiment with human volunteer subjects. The 94 per cent increase in excretion of radioactivity attributable to ingestion of food prior to test is statistically significant ($P < 0.001$).

In the second experiment (Table I), the ratio of excretion of radioactivity in the prefed group (eleven subjects) to the fasted group (twelve subjects) was found to be 1.96. The 96 per cent increase attributable to feeding is statistically significant ($P < 0.001$).

TABLE II
Studies in Rats
(Radioactivity* in Livers and Kidneys)

Study	Liver			Kidneys		
	Fasted	Fed	Ratio	Fasted	Fed	Ratio
A (5/5)†	0.72	1.49	2.07	1.50	2.90	1.93
B (5/5)	1.05	2.24	2.13	1.17	3.11	2.66
C (6/6)	1.27	2.44	1.92	2.53	4.79	1.89
D (6/5)	1.08	2.33	2.16	4.01	7.73	1.93
Mean			2.07			2.10

* Radioactivity expressed as geometric mean millimicrograms of labeled vitamin B_{12} in the organ.
† Numbers in parentheses correspond to numbers of subjects (rats) in fasted and fed groups, respectively.

Four separate rat studies were completed in which the radioactivity in liver and kidneys was determined. Table II is a summary of the data and the meaningful ratios. With respect to both liver and kidney values, the ratios did not differ significantly from experiment to experiment. The mean ratios, 2.07 for liver levels and 2.10 for kidney levels, correspond to increases in the fed animals greater than 100 per cent over the fasted animals. These are statistically very highly significant ($P < 0.001$).

In experiment A, the fecal excretion of radioactivity was determined. In the fed group this excretion was 30 per cent less than in the fasted group. This difference is compatible with the hypothesis that feeding enhances absorption, but the data are too few to be statistically significant.

COMMENTS

The vitamin B_{12} molecule is poorly absorbed from the gastrointestinal tract of even clinically healthy subjects. Numerous attempts have been made to increase the absorption of the orally administered vitamin and the literature contains conflicting reports on the measure of success attained.

Unfortunately, the absorption of vitamin B_{12} is extremely difficult to measure explicitly, and substantially all the conclusions referred to, including those deduced from this communication, rest on inferences drawn from more readily measurable data. It is logical to assume that the absorption of the vitamin B_{12} molecule parallels the absorption by tissues or

urinary excretion of the radioactive tag, usually cobalt^{58} or cobalt^{60} in labeled vitamin B_{12}, and there are at least a few experiments which support the validity of inferences so drawn.

The majority of the studies rely for their conclusiveness on the ability of the person carrying out the experiment to make certain statements about the formal statistical significances of the reported findings. Almost uniformly, however, these statements beg the question whether the observed differences are physiologically meaningful and indeed we have little or nothing in the way of yardsticks by which we can measure the physiologic significance of any of the differences reported. No doubt the best criteria are those seen in studies of patients with pernicious anemia in whom reticulocytosis, increases in hemoglobin level and clinical improvement are less obscure evidences of beneficient changes. However, the population available for such studies is small and the applicability of findings in such populations to the population at large is questionable, because of the known defect in intrinsic factor output in the patient with pernicious anemia.

Nevertheless, and even with all these limitations in mind, the data presented in this communication demonstrate quite clearly that there is a statistically significant increase in the apparent absorption of vitamin B_{12} from the gastrointestinal tract following the oral administration of the vitamin to clinically healthy human subjects and to rats, when the vitamin is offered after feeding rather than on an empty stomach. Curiously, the conclusions reported herein with respect to human subjects are contradictory to those reported earlier by Swenseid et al.,[3] and this difference still requires reconciliation. Direct comparison of the studies cannot be made because of differences in methods and dose range. However, despite their conclusions, Swenseid et al., did show an enhancement of absorption by food at a 0.5 μg. dose and speculate that secretion of intrinsic factor may be stimulated by food.

SUMMARY

Data involving the measurement of absorbed radioactivity following the oral administration of labeled vitamin B_{12} are presented which show greater apparent absorption of the vitamin in prefed human subjects or rats, using as a criteria urinary excretion in man and organ (liver and kidneys) uptake or excretion in rats.

ACKNOWLEDGMENT

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How Much Folic Acid Is Safe in Pernicious Anemia?

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In 1931 Wills[1] described a macrocytic anemia in Hindu women which responded to therapy with autolyzed yeast. This observation initiated a series of investigations which culminated in the discovery in the early 1940's of a new group of growth factors with similar chemical and biologic properties.[2–6] Shortly thereafter substances such as the L. casei factor, vitamin M and vitamin B_c were recognized to be probably identical, and chemical identification of the active principle, pteroylglutamic acid, more commonly known as folic acid, soon followed.[7–10]

Even before chemical identification of folic acid, deficiency states cured by crude concentrates of yeast and liver had been described in men, monkeys, rats, insects and microorganisms.[11,12] The first clinical studies employing synthetic folic acid were reported in 1945,[13] and subsequently there were numerous publications dealing with the therapeutic value of folic acid.[14–17] Initially, folic acid appeared to be a new potent hematologic principle effective in megaloblastic anemias of all types. However, its failure to ameliorate or prevent the neurologic complications of Addisonian anemia soon became evident, and its use in this disease was condemned.[18–21]

FOLIC ACID IN MULTIVITAMIN PREPARATIONS

Soon after its discovery, folic acid was added to a number of multivitamin preparations, usually, but not always, in a dosage lower than that used therapeutically. Undoubtedly, the rationale for this measure included the assumption that folic acid may be taken for long periods with complete safety and that it is a vitamin essential for man which may be inadequate in the diet. However, clear distinction between its use as a vitamin supplement (an adjunct to diet) and as a hematinic (meant to cure anemia) was often lacking. Recognition of the inherent danger of masking pernicious anemia by inadvertent folic acid therapy led to a reduction of the dosage of folic acid in many multivitamin preparations available for general prophylactic use (0.25 to 0.5 mg.). It also led to considerable controversy as to whether such amounts may be safely incorporated in vitamin preparations which may be prescribed for long periods.

Proponents have held that modest amounts of folic acid (circa 0.4 mg. per day) are innocuous, since greater amounts are taken in the normal diet. They point out that such "physiologic" dosages fail to induce a hematologic remission in pernicious anemia, but will prevent or relieve anemias due to folic acid deficiency.[22,23]

Advocates of removal of folic acid from preparations not intended for specific therapy question whether any dosage may be deemed safe as long as multivitamin preparations containing folic acid are readily available to the patient who may regulate his own dosage and may take the medication for protracted periods.[24,25] They point to recent instances of permanent neurologic damage in patients with mild or no anemia who were taking folic acid as a combined supplement.[26–28] Whether or not simultaneous vitamin B_{12} therapy would have prevented such catastrophies is not pertinent to their argument for they emphasize the danger of delayed recognition and diagnosis of pernicious anemia.

Because of this controversy, the Food and Drug Administration recently recommended

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that vitamin preparations containing more than 400 μg. of folic acid in a daily dose be sold only on prescription.[29] The critical factor in consideration of safety is whether or not amounts of 400 μg. or less may be therapeutically active* in pernicious anemia, and it is on this point that "to add or not to add" folic acid must be judged. At present, neither the effects of "physiologic (250 to 500 μg.) nor "minute" (25 to 250 μg.) daily doses of folic acid have been studied extensively in patients with megaloblastic anemias, particularly pernicious anemia. Consequently, any attempt to assess the safety of incorporating microdoses of folic acid in vitamin preparations at this time must be based on indirect evidence.

MICRODOSES IN TROPICAL SPRUE

Clinical experience has shown that folic acid is specifically indicated in megaloblastic anemias associated with sprue, infancy, malnutrition and pregnancy.[30–34] Observations made in the treatment of these anemias with folic acid has contributed to our knowledge of the therapeutic effect of this vitamin and to man's daily need for it. Originally, 100 to 200 mg. daily were considered necessary for treatment; later, smaller amounts were found satisfactory. At present, 5 to 20 mg. daily of folic acid are recommended for the treatment of megaloblastic anemias resulting from a deficiency of this vitamin. However, Woodruff and his associates[35] found that as little as 200 to 500 μg. daily of the vitamin, given orally, produced a remission in infants with megaloblastic anemias and similar dosages, 125 to 250 μg. daily, have been used to relieve megaloblastic anemias in patients with cirrhosis and scurvy.[36,37]

These observations with microdoses of folic acid suggested that very little of the vitamin may be required by patients with anemias resulting from a deficiency of folic acid. In order to assess this possibility, we recently used "minute" doses, 25 μg. daily to treat thirty patients with the megaloblastic anemia of tropical sprue. Clinically, these patients were similar to those described by Spies et al.[31] in one of the original treatises on folic acid therapy. These patients were all maintained on a diet of native foods containing ample dietary folic acid-like activity† for three or four weeks without benefit. Thereafter they were treated orally with 25 μg. daily of folic acid, an amount of approximating the folic acid content of early liver extracts used in the treatment of sprue.[38] If a hematologic response was not evident within ten to fifteen days, the daily dosage of folic acid was increased by increments of 25 or 50 μg. every seven days.

The patients were then classified in three groups according to their hematologic response. Group A consisted of eleven patients who responded to 25 μg. of folic acid daily (Fig. 1 and 2). Group B consisted of eight patients who responded hematologically when the daily dosage of folic acid was increased to 100 or 250 μg. daily. In some cases, in both groups, the reticulocyte response was suboptimal and the reversion from megaloblastic to normoblastic erythroid maturation was delayed. Group C consisted of eleven patients who failed to improve on the initial 25 μg. doses; six of these patients failed to respond to increased doses of folic acid ranging from 100 to 250 μg. daily; one patient failed to respond to daily doses of 250 μg. orally and 500 μg. parenterally.

VITAMIN B_{12} LEVELS

One of the factors suspected of contributing to the different hematologic responses of these superficially similar patients was a coexistent deficiency of vitamin B_{12}. In tropical sprue, as in pernicious anemia, the serum vitamin B_{12} level is abnormal, the intestinal absorption of vitamin B_{12} is impaired, even in many asymptomatic treated patients, and either folic acid or vitamin B_{12} can be used to induce a hematologic remission. The serum vitamin B_{12} levels of the patients in group A were found to range from 50 to 200 μμg. per ml., mean 110 μμg. per ml. (Euglena gracilis: normal, 400 ± 150 μμg. per ml.) The serum vitamin B_{12} levels of the eight patients in

* Active should not be confused with effective, as effectiveness is a subjective term qualified differently by different investigators.

† 50 to 100 μg. of "free" folic acid-like activity; 1,000 to 1,500 μg. of "bound" folic acid-like activity daily.

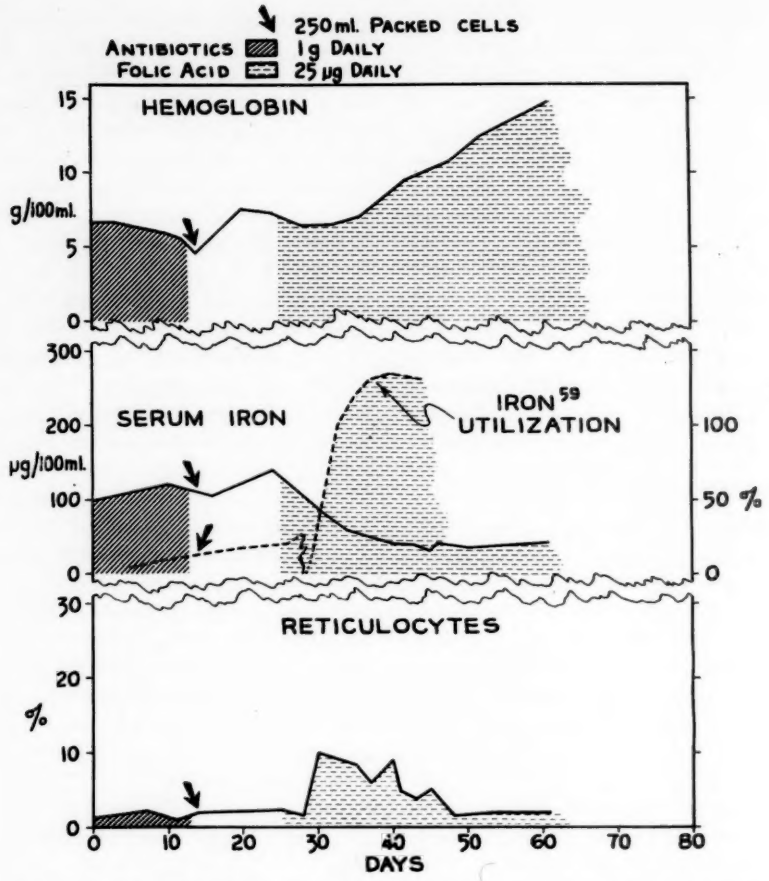

FIG. 1. In this patient, 25 µg. doses of folic acid resulted in a good clinical and hematologic response. The serum iron level fell and the incorporation of Fe^{59} by the red blood cells increased rapidly following the administration of folic acid. The reticulocyte response in this instance was suboptimal.

group B ranged from 25 to 75 µµg. per ml., mean 65 µµg. per ml. The serum vitamin B_{12} levels of the patients in group C ranged from 15 to 90 µµg. per ml., mean 50 µµg. per ml. None of the patients with serum vitamin levels below 50 µµg. per ml. responded to therapy with 25 µg. doses of folic acid, but many responded hematologically to the slightly larger doses (Fig. 3). The patients in group C who failed to respond to the initial dose and to the increased doses of folic acid subsequently responded within eight to fourteen days to parenterally administered vitamin B_{12}, 1 µg. daily. Therapeutically this was further evidence in support of a coexistent vitamin B_{12} deficiency in sprue which may have impeded the hematologic response to folic acid. However, tropical sprue may be complicated by other factors, such as parasitism, hypoproteinemia, and severe malnutrition, any of which may have impaired a response to folic acid.

COMMENTS

The question of the general advisability of incorporating "physiologic" amounts of folic acid in multivitamin preparations cannot be answered from data derived in a study of tropical sprue. This problem concerns the

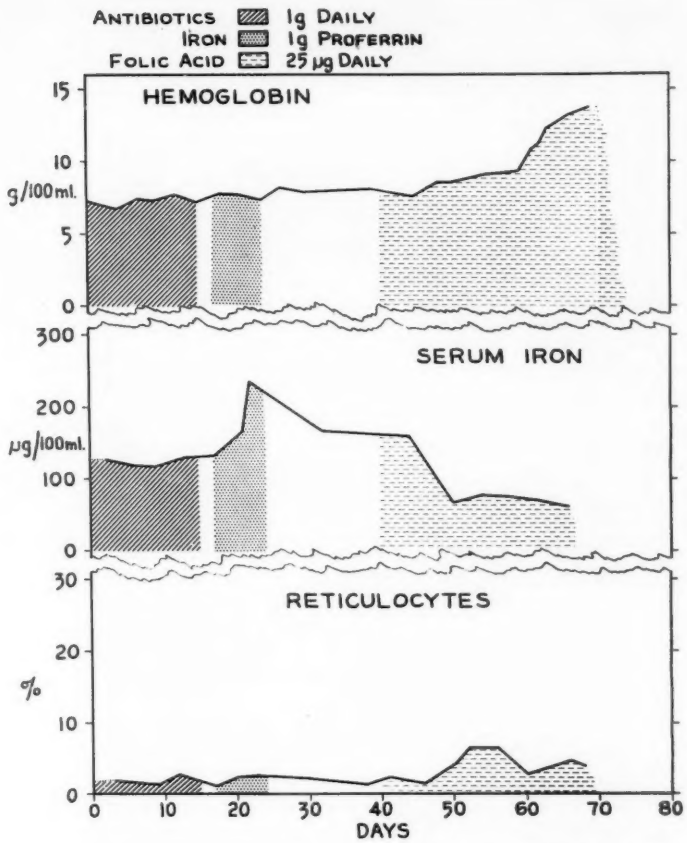

FIG. 2. Clinical improvement was evident in this patient several days after folic acid was administered. Shortly thereafter the patient's hemoglobin level began to rise and eventually reached normal value. A prominent reticulocyte response was never observed. However, in most patients treated successfully with 25 µg. of folic acid daily, the reticulocyte peak varied from 8 to 28 per cent.

safety of multivitamin preparations in the hands of individuals with unrecognized pernicious anemias and at present our knowledge of the action of "physiologic" doses of folic acid in this disease is limited. The concept of the use of "physiologic" doses of folic acid was ingeniously devised to differentiate megaloblastic anemia etiologically.[39] Three patients with pernicious anemia were given 400 µg. of folic acid parenterally daily for ten days. They failed to respond hematologically whereas three patients with megaloblastic anemias resulting from a deficiency of folic acid responded well to the same form of therapy. Unfortunately, this experimental situation was not analogous to the individual with unrecognized pernicious anemia taking a multivitamin preparation for many weeks or months.[25] It did not provide conclusive proof that 0.2 to 0.5 mg. of folic acid daily of this vitamin is harmless in pernicious anemia. It did not prove that such doses are insufficient to mask an incipient megaloblastic anemia while allowing the neurologic lesion of pernicious anemia to progress. Experience gained in the treatment of tropical sprue, albeit not applicable to pernicious anemia, showed that 0.1 to 0.25 mg. of folic acid daily can be active

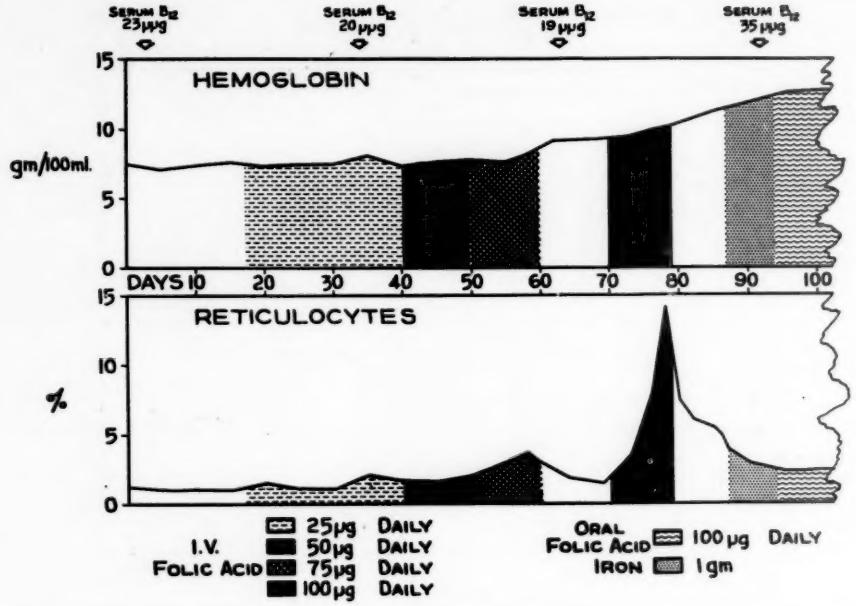

FIG. 3. This patient had a nine year history of sprue and had suffered three previous relapses as a result of discontinuing folic acid therapy. His serum vitamin B_{12} level was found to be low one year prior to his fourth relapse. Oral therapy with 25 µg. folic acid daily did not improve the patient or his blood picture. Thereafter the daily dose of folic acid was given parenterally and was increased by increments of 25 µg. daily every ten days. A hematologic response occurred when the titered dosage reached 100 µg. daily.

in the presence of serum levels consistent with an early deficiency of vitamin B_{12}.[40] These results cannot be dismissed until more patients with Addisonian anemia are treated for more prolonged periods with "physiologic" doses of folic acid. One must question the equanimity with which "physiologic" or "safe" dosage has been recommended. It was for a diagnostic not for a therapeutic purpose that "physiologic" doses were recommended in pernicious anemia.

Considerations of the value of including folic acid in multivitamin preparations must weigh the potential general benefit of the community against the danger to patients with unrecognized vitamin B_{12} deficiency. In tropical areas, particularly in underdeveloped countries where malnutrition is prevalent and medical care inadequate, the incorporation of folic acid into multivitamin preparations may be advisable. Megaloblastic anemias resulting from folic acid deficiency are extremely common in these areas whereas neurologic deterioration due to a deficiency of vitamin B_{12} is exceedingly rare. Here, the general welfare of the populace weighs heavily in favor of such a measure, but in medically advanced communities with more adequate diagnostic facilities this argument loses weight. In such areas, little harm would result from the removal of folic acid from multivitamin preparations until the issue of the effect of microdoses of folic acid in pernicious anemia is solved. In this instance, the general health of the community would not be seriously compromised in protecting a small segment of the populace.

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EDITORIAL COMMENT

As an editorial response to Dr. Sheehy's remarks, first it may be pointed out that his observations that some patients with the megaloblastic anemia of sprue may respond to very small doses of folic acid provides new and important information as to the minimally effective dose of folic acid. Whether these patients, with their multiple deficiencies, including deficiency of vitamin B_{12}, are especially sensitive to folic acid, and whether preliminary therapy with antibiotics may have potentiated their responsiveness, remains to be determined. We look forward to further study of this phenomenon from Dr. Sheehy and his collaborators.

These studies do not particularly bear on the question of what constitutes a "safe" dose level of folic acid in patients with vitamin B_{12} deficiency. The solution to that question must be derived from long-term studies of patients with pure vitamin B_{12} deficiency (pernicious anemia) placed on low doses (i.e., 0.4 mg. or less) of folic acid and compared to a similar group of patients not so treated.

One can make a reasonable argument based on available evidence either for or against the inclusion of small amounts of folic acid (we have recommended 0.1 or 0.2 mg. daily)* in multivitamin preparations. If it can be shown by Dr. Sheehy or others that considerably smaller doses do indeed afford reliable therapy for folic acid deficiency, any potential risk to patients with pernicious anemia could presumably be still further reduced by adopting a lower dose level of folic acid for general use. However, it should be realized that deficiency of folic acid is common; at the Boston City Hospital, at least, it is considerably more frequent than vitamin B_{12} deficiency. In our opinion, it would be a pity to deny this nutrient to the many because of the unproved possibility that small doses of folic acid taken for a long period might mask the clinical picture of some unrecognized cases of pernicious anemia. At present it would seem the lesser of two risks to permit small amounts of folic acid to be included in multivitamins preparations pending further information, than to ban such use prematurely.

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Effects of Amino Acid Supplements on the Nutritive Value of Maize Protein for Human Adults

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Maize provides the main article of food for the Bantu of Southern Africa,[1] in large parts of Central America and some other regions of the world.[2] Its nutritional importance in these populations is heightened by their small consumption of animal protein foodstuffs. A knowledge of which amino acids will improve the quality of maize protein for man would provide a basis for choosing natural protein supplements for sections of these populations that are shown liable to protein malnutrition.

Average amino acid analyses of whole maize show a relative deficiency of tryptophan and lysine, whether compared with estimates of the requirements of amino acids for man,[3] or the pattern of essential amino acids in hens' egg[4] or human milk.[5]

The order in which amino acids appear deficient by comparing chemical composition with standard amino acid patterns is not always borne out on biologic testing. Determination of requirements by experiments with mixtures of pure amino acids necessitates several artificial conditions. In addition, there is evidence that some amino acids are poorly released during digestion of maize[6,7] or zein.[8,9] It is also likely that the excessive proportion of leucine in maize produces an amino acid imbalance, and increases the need for isoleucine[10] and possibly valine.[11,12]

With rats it has been consistently found that the nutritive value of maize is improved by the addition of lysine *plus* tryptophan.[13–17] The available evidence[10,16–19] indicates that the third and fourth limiting amino acids are isoleucine and threonine, their respective order is not clear because isoleucine has not been added to the diets of animals fed maize supplemented with only lysine and tryptophan. Sauberlich's studies[16] suggest that valine is even less limiting and that there is no deficiency of methionine in maize.

These results in rats cannot be applied directly to human nutrition. With soya,[20] casein,[21] rice[22] and groundnuts[23] amino acid supplementation did not give the same results in man as have been found in experimental animals. These interspecies differences may be partly explained by the larger proportion of methionine required by rats,[24] but it is possible that the difficulties in controlling human experiments were responsible as well.

EXPERIMENTAL

We have compared nitrogen balances in men on maize diets with the same subjects on diets changed only by substitution of amino acids for an isonitrogenous amount of the maize protein. The first supplement tried was lysine plus tryptophan plus isoleucine. It was chosen because we expected from the published reports of animal experiments (*vide supra*) that it would be nearly optimal. It was tested under several different sets of experimental conditions in order to find which conditions would make the subjects' nitrogen balance most sensitive

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TABLE
Effects on Nitrogen Balance of Supplementing Maize

Subject and Age (yr.)	Diagnosis	Conditions: Experiment No.	Protein Intake (gm./kg.)	Days for Adjustment	% Protein Intake not from Maize*	Calorie Intake (per kg.)
I. A., 34	Convalescent toxic psychosis	8	0.47	33	10	44
M. P., 45	Old (L) hemiparesis, essential hypertension	9	0.45	15	10	36
J. M., 56	Convalescent subdural haematoma	10	1.01	4	6	58
K. S., 23	Simple schizophrenia	11	0.93	9	4	41
		17	0.86	7	4	38
J. W., 22	Fibrositis	14	1.02	12	5	45
P. N., 24	Feeble minded	13	0.99	11	0	46
		16	0.95	102‡	0	40
		23§	0.87	107	0	40
J. Ph., 46	Old weakness right leg	15a	0.93	8	5	39
		15b	0.89	27	5	54

* All from pumpkin and/or squash (both Cucurbita Pepo L.)

† Mean day-to-day weight change = $[\sum W_{(r+1)} - W_r]/n$

where Wr = weight on one day
$W_{(r+1)}$ = weight the next day
n = no. of days per period.

to differences in the quality of protein. Whichever conditions were chosen, they were kept constant throughout an experiment.

We found that this supplement had a demonstrable effect under most conditions. We then continued testing with other combinations of amino acids and have obtained some indication of the order in which amino acids are deficient in maize for human nutrition.

Eight male subjects participated in this study. Six were Bantu and two Cape Coloured. They ranged in age from twenty-two to fifty-six years.

I
with Lysine, plus Tryptophan plus Isoleucine

Mean Day-to-Day Weight Change (kg./day)†	Other	Nitrogen Balance (mean) gm./day					
		A		B		C	
		On Maize Alone		On Maize plus lysine tryptophan and Isoleucine		Difference (B-A)	
−0.014	...	+0.014		+0.271 +0.234	+0.253	+0.239	
+0.007	...	+0.030 −0.192	−0.081	+0.478 +0.277 +0.394	+0.383	+0.464	
+0.120	...	+2.401 +2.405	+2.403	+3.404		+1.001	
−0.022	Durabolin, 25 mg. intramuscularly every third day	+0.422 +0.319 +0.873	+0.538	+1.078 +0.869	+0.974	+0.436	+0.575
+0.017	...	−0.410 −0.014	−0.212	+0.502		+0.714	
0	In bed	+0.640		+1.000		+0.360	
+0.026	...	+0.600 +0.794 +0.759	+0.718	+1.281 +1.342	+1.312	+0.594	+0.454
0	...	+0.068		+0.379		+0.311	
+0.011	...	+0.438		+0.895		+0.457	
−0.057	More active than the other subjects	−0.266		−0.232		+0.034	+0.343
+0.074		+0.770 +1.155	+0.963	+1.615		+0.652	
				Mean			+0.491

‡ But a course of Nilevar was stopped immediately before the first test period (maize plus amino acids).
§ DL-tryptophan used.

Most of them had mental or static physical disabilities. It was considered that all should have normal protein metabolism. Their diagnoses are recorded in Table I. They were accustomed to a typical Bantu diet containing large amounts of maize. Five of them had eaten mixed hospital diets for a few weeks before the commencement of the study. None showed any clinical sign of malnutrition. All had normal blood urea levels. Their initial serum albumin levels ranged from 3.6 to 4.4 gm./100 ml. and their total serum globulins from 2.4 to 3.2 gm./100 ml.

They lived in a metabolic ward. Their activity was limited and very nearly constant. Continuous

TABLE II

Effects on Nitrogen Balance of Supplementing Maize with Lysine plus Tryptophan

Subject	Experiment No.	Protein Intake not from Maize	Remarks	Nitrogen Balance (mean) gm./day		
				A	B	C
				On Maize alone	On Maize plus Lysine and Tryptophan	Differences (B-A)
K. S.	17	Pumpkin 4%	...	+0.043	+0.878	+0.835
P. N.	13a	0	...	+0.759	+1.175	+0.416
	13b	0	...	+0.374	+1.121	+0.747
	16	0	...	+0.759	+1.322	+0.563
	19	0	...	+0.315	+1.170	+0.855
	23	0	DL-tryptophan periods 9 days apart	+0.438	+0.816	+0.378
	23	0	DL-tryptophan	+0.484	+0.687	+0.203
M. P.	3	Bread 12% Pumpkin plus squash 10%	DL-isoleucine, DL-tryptophan	+0.711	+1.442*	+0.731
	18	Pumpkin 5%	...	+0.486	+1.390	+0.904
	22	Pumpkin 6%	DL-tryptophan	+0.701	+0.828	+0.127
	29a	Pumpkin 4%	...	−0.181	−0.221	−0.040
	29a	Pumpkin 4%	DL-tryptophan	+0.428	+0.219	−0.209
	29b	Pumpkin 4%	Testosterone propionate 50 mg. intramuscularly on alternate days	+1.683*	+2.624	+0.936
					Mean	+0.496

* Mean of two balances, before and after the other diet.

nitrogen balances were determined. Prolonged adjustment periods were allowed before the results of the different diets were compared. Each subject then served as his own control and the results are a series of pairs of nitrogen balances on the two particular diets compared. Periods were usually nine days long because it was found that faecal nitrogen was less variable than with shorter periods. The two diets being compared were fed in consecutive periods.

Nitrogen was measured by a standard semi-micro Kjeldahl method, using HgO and K_2SO_4 as digestion catalysts. Urinary nitrogen was determined daily (in duplicate) and checked by measuring creatinine excretion. Carmine, 1.2 gm., was given to separate the stools. Faeces were frozen immediately after they were collected. At the end of each period they were mixed in three-day lots and nitrogen was determined on three aliquots of undried faeces from each three-day pool.

In most experiments the protein intake was about 0.9 gm./kg. body weight, with 90 to 100 per cent of it from maize. In two experiments it was approximately 0.5 gm./kg. Maize provided the bulk of the caloric intake. The subjects were able to eat large amounts of maize meal—500 to 600 gm./day. The diets were very simple. In most experiments the only other items were pumpkin and squash (both varieties of cucurbita Pepo L.) sucrose, sodium chloride, Sucaryl,® black coffee and tablets of iron, calcium and vitamins.* (In a few experiments white bread, margarine or sunflower seed oil was also given.) The caloric intake was kept constant throughout each experiment. It was usually adequate to maintain body weight or produce a slight increase. In a few experiments anabolic conditions

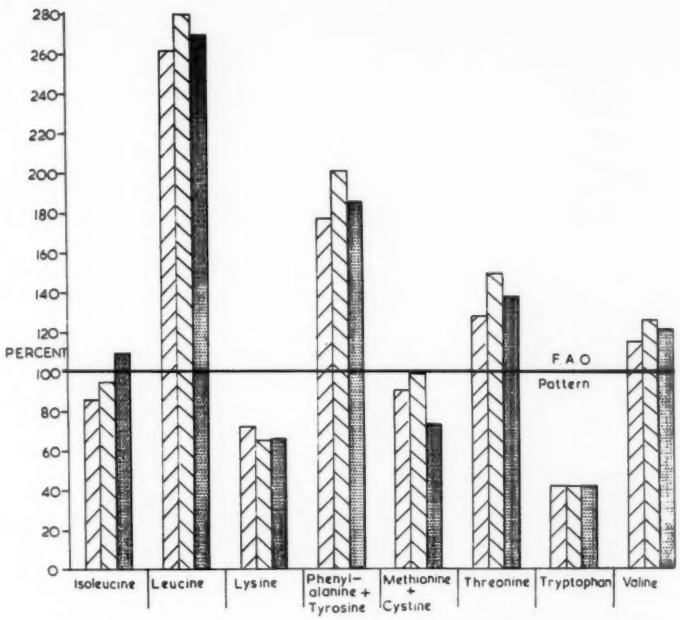

FIG. 1. Aminograms of the maize used (first batch left hand bars, second batch middle bars) and the average values for maize by Orr and Watt[3] (right hand bars) all in mg. amino acid/1 gm. nitrogen and expressed as percentages of the F.A.O. provisional pattern of essential amino acids requirements.[3]

were produced by feeding excessive calories[25] or giving androgens throughout (Tables I and II.)

Two large batches of whole South African maize meal (97 per cent extraction) were specially milled† The meal was kept at 32° to 40°F.‡ until a week before it was eaten. The first batch was used during 1957 and 1958. It contained (nitrogen × 6.25) 9.4 per cent protein (undried). The second batch contained 9 per cent protein and was used throughout 1959. Full proximate analysis of the meal§ showed that it did not differ notably from other South African samples.[26] Complete amino acid analyses‖ of both batches by the method of Moore and Stein[27] are compared in Figure 1 with world average values.[3] The maize meal was always served as porridge, cooked in a double boiler. To calculate the nitrogen intake six replicate Kjeldahl analyses were made of the maize meal every week. As we could expect the maize to be of nearly constant composition this largely served as an additional check on the standardization of the Kjeldahl method. Other foods were analysed every week at first and every two to four weeks thereafter.

Except for methionine the amino acids were given in the L-form unless otherwise noted in the tables. One-third of the daily dose was taken in aqueous solution during each meal. The amounts given as supplements were chosen before amino acid analysis of the maize and before publication of the FAO provisional amino acid pattern.[3] For lysine and tryptophan, Rose's safe requirements[28] of the L-amino acid were usually used. Smaller amounts

* The vitamin tablets provided per day:

Vitamin A	10,000 I.U.
Thiamine	4 mg.
Riboflavine	5 mg.
Niacin	40 mg.
Calcium pantothenate	1 mg.
Pyridoxine	1 mg.
Vitamin B_{12}	20 μg.
Vitamin C	50 mg.
Calciferol	800 I.U.

† Vereeniging Consolidated Mills Ltd., Cape Town, South Africa.

‡ We are indebted to Table Bay Cold Storage Company, Ltd.. Cape Town, South Africa.

§ Mr. A. S. Wehmeyer of the National Nutrition Research Institute, Pretoria, South Africa.

‖ Dr. S. G. Wiechers of the Fishing Industry Research Institute, Cape Town, South Africa.

TABLE III
Changes in the Amino Acid Composition of the Diets Produced by Amino Acid Supplements

Amino Acid	Amino Acids (mg./1 gm. nitrogen)					
	Unsupplemented Maize (mean of 1st and 2nd batches)	Supplemented		F.A.O. Provisional Amino Acid Pattern[3]	Whole Hens' Egg[3]	Cows' Milk[3]
		Average When Protein Intake 0.9 gm./kg.	On Low Protein Diets (0.45 gm./kg.*)			
Lysine	184	378	605	270	396	496
Tryptophan	38	101	168	90	106	90
Isoleucine	244	369	464	270	428	407
Methionine plus cystine	256	371	...	270	342	211

* These values were the highest produced. They only apply to two experiments.

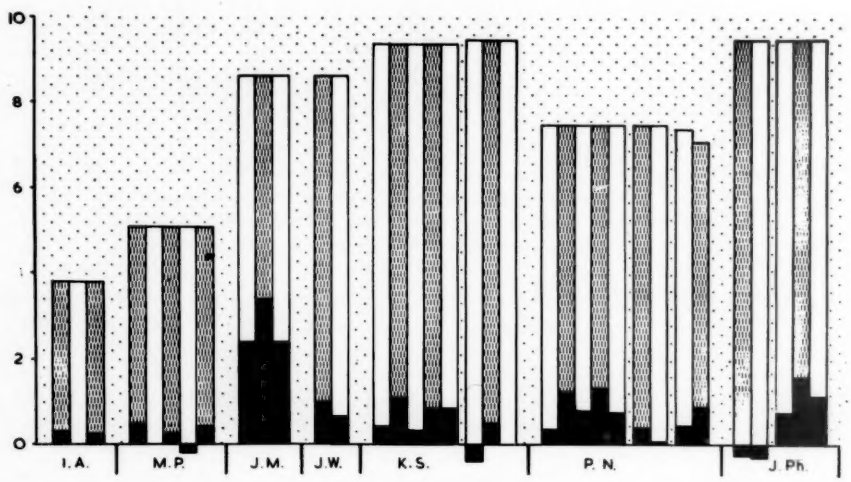

FIG. 2. Nitrogen balances on maize with and without lysine plus tryptophan plus isoleucine. Each bar represents a six- to twelve-day period. The tops of the bars indicate the nitrogen intake. Shading indicates that amino acids were given. Nitrogen balances are shown by solid black areas. The narrow gaps between the bars separate the different experiments for K. S., P. N. and J. Ph. In each experiment the order of feeding the diets is from left to right.

of isoleucine and methionine were given.* When supplements of three amino acids were given they replaced together 0.25 to 0.71 per cent of total calories and 3.7 to 7.6 per cent of the nitrogen intake (10 per cent with low protein intakes). When only one or two amino acids were given, they made up a correspondingly smaller proportion of the diet. The changes in the dietary aminogram produced by these supplements are shown in Table III.

* *viz.* 1.6 gm./day L-lysine (2 gm./Darvyl L-lysine monohydrochloride [du Pont]); 0.5 gm./day L-tryptophan; 1.0 gm./day L-isoleucine; 1.0 gm./day DL-methionine (crystalline Meonine® [Wyeth]).

RESULTS

Effect of Supplementing Maize with Lysine plus Tryptophan plus Isoleucine

The balance results are illustrated in Figure 2 and experimental details are recorded in Table I. There was a total of 161 test days with the subjects on maize alone and 135 days

with the subjects on maize plus lysine, tryptophan and isoleucine. In all seven subjects the mean nitrogen balances increased while they were receiving the supplement. This is statistically significant ($P < 0.02$) by a two-tailed sign test.

It is interesting that this increase occurred in experiments 13, 14, 16 and 23 because the subjects already had small positive balances on maize alone and the conditions were static as judged by the trivial changes in weight. The effect of supplementation was least in experiment 15a in which the caloric intake was inadequate. The largest effect was observed in subject J. M. who received excessive calories; he gained weight rapidly and his skinfold thickness increased. Use of Durabolin® (19-norandrostenolone phenylpropionate) did not appear to sensitize subject K. S. to a change in protein quality. His nitrogen balances were higher in the experiment on Durabolin but he did not gain weight.

In critical evaluation of these results two influences must be considered. First, the order of feeding the diets might have had an effect. After the standard week for adjustment following a change of nitrogen intake, we have noticed a tendency for the balance to continue to drift in the same direction as during adjustment for up to a few weeks. An example of this phenomenon is illustrated in Figure 3. An effect from order of feeding was eliminated as follows: periods with and without amino acids were adjacent and, when multiple, alternating (Fig. 2). One of the two diets was fed both before and after the other in seven of the eleven experiments. In the remaining four experiments the order favoured the maize diet in three.

Secondly, on the supplemented diets approximately 5 per cent of the nitrogen intake was replaced by pure amino acids, which should be better absorbed than maize. It is possible that the increased nitrogen balance during supplementation resulted from improved absorption rather than a higher biologic value. In fact, the mean increase of nitrogen balance with amino acids for the seven subjects was 0.49 gm. nitrogen day, while the difference in mean faecal nitrogen between the two diets

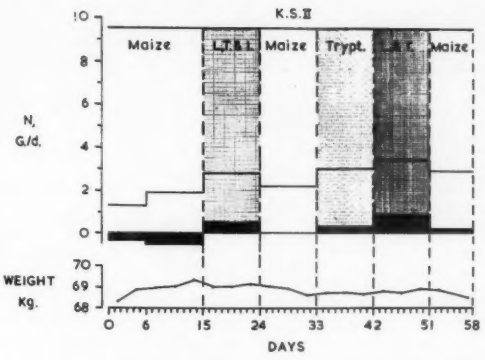

FIG. 3. In this subject the experimental diet was started one day before day 0. The first seven days were allowed for adjustment. Note how the mean urinary nitrogens are less in each of the three succeeding periods on maize. The nitrogen balances on maize consequently increase (this effect is masked in the first two periods by the small faecal nitrogen from days 1 to 6). The pattern suggests that the subject's nitrogen intake before admission was higher than during the experiment. (His dietary history was unreliable.) The top horizontal line represents the intake: the next line down separates urinary nitrogen above from faecal nitrogen below. Balance is represented by solid black, above the zero line if positive. L = lysine; T = tryptophan; I = isoleucine; all as supplements to maize.

was only 0.06 gm. nitrogen day in favour of the supplemented diet. When the differences in faecal nitrogen are subtracted from the differences between the balances in each subject, the results are still in favour of maize plus lysine, tryptophan and isoleucine in all seven.

Effect of Adding Lysine plus Tryptophan to Maize

The effect of a supplement of lysine plus tryptophan was investigated next because in reported animal experiments these have been the first two limiting amino acids in maize. This two amino acid supplement was given to three of the same subjects in a total of thirteen pairs of adjacent nine-day nitrogen balance studies. The results are shown in Table II. In all experiments the protein intake was near 0.9 gm./kg. in the different trials. The caloric intake (35 to 45/kg. in the different experiments) appeared adequate. There was a small gain of 0.009 to 0.043 kg. body weight/day in all but three experiments. In experi-

TABLE IV
Effect of Adding Isoleucine to Maize plus Lysine and Tryptophan

Subject	Experiment No.	% Protein Intake not from Maize	Remarks	Nitrogen Balance (mean), gm./day		
				A	B	C
				On Maize plus Lysine and Tryptophan	On Maize plus Lysine, Tryptophan and Isoleucine	Difference (B-A)
M. P.	3	Bread 12 Cucurbitae 10	Static conditions, DL-isoleucine and DL-tryptophan	+1.662	+1.855	+0.193
	29	Pumpkin 4	Nitrogen intake reduced nine days previously; feeding order favoured maize plus lysine and tryptophan	−0.221	+0.018	+0.239
J. P.*	4	Bread 12 Cucurbitae 10	Excessive calorie intake; gained 0.119 kg. body weight per day; DL-isoleucine and DL-tryptophan	+2.284	+2.423	+0.12
P. N.	13	0	Two periods separated by nine days on maize; static conditions	+1.175	+1.342	+0.167
	23	0	Static conditions; DL-tryptophan	+0.816	+0.895	+0.079
					Mean	+0.161

* Subject not appearing in Tables II or III, a thirty year old Bantu male with weakness of both legs from corticospinal degeneration. Sphincter control was preserved.

ments 17 and 22 there was a slight weight loss of 0.035 and 0.004 kg./day, respectively. In experiment 29b anabolic conditions were produced by giving the subject 50 mg. testosterone proportionate intramuscularly on alternate days throughout. This resulted in a mean day-to-day weight gain of 0.102 kg./day and a higher level of nitrogen balance.

The time for adjustment to the nitrogen intake before testing was thirty-five days or longer in all experiments except experiment 29 in which it was eighteen days. In experiments 3 and 29b one diet was given before and after the other. In the remaining experiments the order of feeding would have tended to favour the maize diet in seven experiments and maize plus lysine and tryptophan in four. However, in many experiments the time for adjustment to the nitrogen intake was so long (up to 146 days) that no drift of nitrogen balance was discernible.

The nitrogen balance was higher on the maize plus lysine and tryptophan diet in three of three subjects and in eleven of thirteen paired balance studies in these three subjects. The largest increase in nitrogen balance with supplementation occurred in experiment 29b made under anabolic conditions produced by testos-

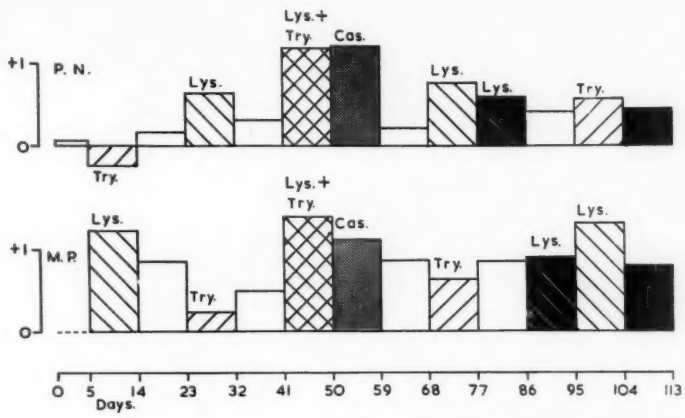

FIG. 4. Nitrogen balances (mean) in gm./day during a synchronous trial of lysine and tryptophan as single supplements to maize in two subjects. Experimental diets were started three days before day 0. The protein intakes were 0.95 (P. N.) and 0.88 (M. P.) gm./kg. per day. In unsupplemented periods all of P. N.'s nitrogen intake was from maize; M. P. received 95 per cent of his nitrogen intake from maize and 5 per cent from pumpkin. During the experiment, P. N. gained 1.6 kg. (0.015 kg./day) and M. P. gained 1.7 kg. (0.016 kg./day). Lysine replaced 4 per cent (P. N.) and 3 per cent (M. P.) of the nitrogen or 0.3 per cent (P. N.) and 0.25 per cent (M. P.) of the calorie intake. Tryptophan replaced 0.9 per cent (P. N.) and 0.7 per cent (M. P.) of the nitrogen or 0.1 per cent (P. N.) and 0.08 per cent (M. P.) of the calorie intake. Cas. = a period in which 50 per cent of the protein intake from maize was replaced by Casilan. Black areas indicate periods in which the usual multivitamin supplement was omitted.

terone. When the balance differences are corrected for differences between faecal nitrogens they are in favour of the supplemented diet in twelve of thirteen paired studies.

The mean increase in nitrogen balance produced by lysine plus tryptophan in three subjects (0.496 gm./day) was similar to the effect of the three amino acid supplement (Table II) in the same three subjects (mean plus 0.497 gm./day). These values are not strictly comparable because experimental conditions were not the same in the two series of trials. For instance in subject M. P. the three amino acid supplement was tested under the less favourable conditions of a low protein intake. The effect of isoleucine was, however, tested by direct comparison in five paired balance studies.

Effect of Adding Isoleucine to Maize plus Lysine and Tryptophan

Experimental details are shown in Table IV. A total of five tests were made in three subjects. In all of them the protein intake was near 1 gm./kg. body weight. The nitrogen balance increased in five of five pairs. The mean increase appeared to be smaller (plus 0.161 gm./nitrogen/day) than was observed with lysine plus tryptophan (Table II).

Lysine or Tryptophan as Single Amino Acid Supplements to Maize

Lysine and tryptophan were added to maize in turn, singly and together in two subjects. Because small changes in nitrogen balance were expected a block design was used (Fig. 4) to eliminate the effects of small fluctuations in environmental conditions or Kjeldahl standardization. The two subjects were in the metabolic ward at the same time. When lysine was given to one, tryptophan was given to the other and vice versa. Control periods (maize alone) were placed before and after the test periods/in which a single amino acid was

TABLE V
Effect of Lysine Alone on Nitrogen Balance (gm./day)

Subject	A Balance in Control Maize Periods	B Balance on Maize plus Lysine	C Difference in Balances (B-A)	D Balance Difference Corrected for Difference in Faecal Nitrogen
P. N.	+0.242 ab	+0.644	+0.402	+0.490
	+0.306 ab	+0.772	+0.466	+0.478
	*+0.438**	*+0.585**	*+0.147**	*−0.001**
M. P.	+0.851 a	+1.269	+0.418	+0.229
	+0.851 b	+1.311	+0.460	+0.394
	*+0.795**	*+0.900**	*+0.105**	*+0.008**
		Mean	+0.333	+0.266

NOTE: a = period after maize plus lysine; b = period before maize plus lysine; ab = mean of a and b.
* Maize plus lysine and maize control both with vitamin supplement omitted.

added. Both subjects received 0.9 gm. protein/kg. throughout. The caloric intakes were adequate.

Nitrogen balance was increased by *lysine* in six of six tests (Table v). Throughout this work the subjects received a multivitamin supplement providing, inter alia, 40 mg. of niacin daily. We thought it might be of practical importance to try the effect of lysine alone when this extra niacin was not provided. Two comparisons were made, therefore, with the whole multivitamin supplement omitted.* The nitrogen balance was no lower with the subjects on the maize diet but the effect of lysine appeared to be less (Table v).

When the balance changes with the subjects on lysine are corrected for differences in faecal nitrogen between the lysine period and the control period (Table v), the mean increase is slightly less. However, the effect of lysine without extra vitamins disappears when this correction is made.

Tryptophan alone was given in another experiment (Fig. 3) as well as in the experiment illustrated in Figure 4. It caused a reduction in the nitrogen balance in three trials and an increase in two; one of the increases can be explained by a lower faecal nitrogen in the tryptophan period. The mean change in balance as a result of tryptophan was minus 0.111 gm. nitrogen/day. It should be noted that extra vitamins were given throughout all the trials with tryptophan.

Addition of Tryptophan to Maize and Lysine

On the other hand, tryptophan did appear to increase nitrogen balance when added to maize plus lysine. In both subjects in Figure 4 the nitrogen balance was higher on the maize plus lysine and tryptophan diet than in the preceding or following periods on the maize plus lysine diet. It can also be seen in Figure 3 that nitrogen balance increased when lysine was added to a supplement of tryptophan alone.

Comparison of Maize plus Lysine and Tryptophan with Casein plus Maize

In three subjects 50 per cent of the protein intake was replaced by casein ("Casilan," Glaxo). In adjacent periods maize was

* The basal daily niacin intakes were 7 to 10 mg. from maize, mostly not available,[39] plus approximately 5 mg. from coffee.[39]

TABLE VI

Nitrogen Balances Compared on Maize plus Lysine and Tryptophan and on Casein plus Maize

Subject	Nitrogen Balance (gm./day)			Faecal Nitrogen (gm./day)	
	A	B	C		
	On Maize plus Lysine, Tryptophan (and isoleucine*)	On 50% Casein and 50% Maize	Difference (B-A)	On Maize plus Amino Acids	On Casein and Maize
P. N.	1.170	1.203	+0.033	1.667	1.219
M. P.	1.390	1.116	−0.274	2.387	1.372
K. S.	0.869*	1.398	+0.529	1.930	0.505
		Mean	+0.096	1.995	1.032

NOTE: The balances of P. N. and M. P. are also shown in Figure 4.

* K. S. was given isoleucine as well as lysine and tryptophan and received 25 mg. Durabolin intramuscularly every third day.

given with the supplement of lysine and tryptophan. All three comparisons were made at around 0.9 gm. protein intake/kg. There was no appreciable change in weight but Durabolin was given every three days to subject K. S. The nitrogen balance (Table VI) was similar on the two diets despite better absorption of the casein nitrogen.

Other Amino Acids: Methionine

The finding that casein appeared no more effective as a supplement than lysine plus tryptophan suggested that other amino acids are not seriously deficient in maize for human nutrition. However, casein is itself somewhat short of methionine. In addition, comparison of the average amino acid analyses[3,31] of maize with the FAO provisional pattern suggests that the sulphur-containing amino acids are the third limiting amino acids (see right hand bars in Figure 1).

A supplement of methionine plus lysine and tryptophan was given to two subjects on 0.9 gm. protein/kg. from maize. The nitrogen balance was compared with adjacent periods before and after with the subject on maize plus lysine and tryptophan (and isoleucine in one of the subjects). The nitrogen balance fell sharply in the periods with methionine by 0.54 and 0.72 gm./day and was lower then when they received maize alone. The results of one of these two experiments are illustrated in Figure 5.

When amino acid analyses were made, the maize we used was found to have more methionine and cystine than average (Fig. 1), so the concentration of these when extra methionine was given was 37 per cent higher than in the FAO provisional amino acid pattern, but only 9 per cent higher than in egg (Table III).

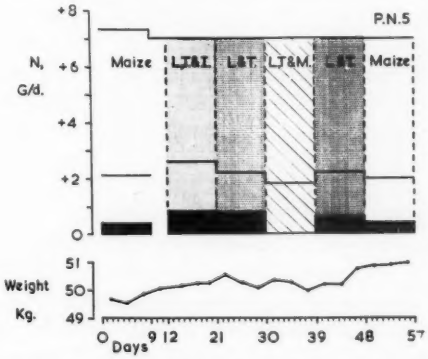

FIG. 5. The effect of adding methionine to a supplement of lysine and tryptophan in a subject who received all his protein intake from maize in the unsupplemented periods. (Method of charting as in Figure 3.)

COMMENTS

We did not at first expect to be able to show differences in protein quality in adults under static conditions if the intake was large enough to give nitrogen equilibrium on maize alone. Hence, in the first two experiments shown in Table 1 a low nitrogen intake was given so that the nitrogen balance with the subjects on unsupplemented maize was negative or zero. In the next two experiments the nitrogen intake was higher but an attempt was made to produce anabolic conditions by giving excessive calories or a testosterone derivative. As all these experiments were made under rather unnatural conditions, it was decided to try an intake of approximately 1 gm./protein/kg. without anabolic stimuli. Although there was usually a small positive nitrogen balance with the subjects receiving maize alone, an increase was readily obtained with supplementation. These conditions were then used in nearly all the subsequent experiments because the percentage of calories from protein was 9 per cent and the diets were such as Bantu adults might really eat at times if other protein foods were not available.

There are two possible reasons for this increase from a small positive to a more positive balance when supplements were added to maize. Either the subjects were subclinically depleted of protein or the nitrogen balance with the subjects on unsupplemented maize was below *true* nitrogen equilibrium when cutaneous and other unmeasured losses of nitrogen are taken into account.

In addition to losses of nitrogen in sweat,[32,33] desquamated skin and hair growth, 15 ml. of blood were taken from our subjects every third day. The latter corresponds to a loss of 0.15 gm./nitrogen/day. Mitchell[34] found the average nitrogen balance of twenty-three normal American men, studied over 200 days, was plus 1.38 gm./day. Our subjects had similar levels when receiving maize supplemented with lysine plus tryptophan or with casein. (It is possible that the lack of difference between the effects of these last two diets was because the nitrogen balance had already reached a ceiling with the amino acid supplement.)

Holmes et al.[35] have recorded that malnourished Africans who had eaten low protein diets all their lives showed a large and very prolonged nitrogen retention when fed on a diet rich in animal protein. We do not believe our subjects were comparable. Their diets at home contained more protein than is usual on the Uganda staple of cassava. Five of them had eaten mixed hospital food for two or more weeks before the experiments began. Unlike Holmes' subjects, ours were not clinically malnourished and in most experiments they did not receive excessive calories or show more than a very gradual gain in weight.

It would seem very unlikely that other protein foods in the experimental diets could have affected our results. The same results were obtained in subject P. N. whose entire protein intake was from maize. In nearly all the other experiments the only other dietary protein was 5 per cent pumpkin and/or squash. In the published aminograms of these cucurbitae[36] the proportions of lysine and tryptophan are a little higher than in maize. There is rather less methionine, threonine and phenylalanine and considerably less leucine.

From our results, the order in which amino acids are limiting in maize (with adequate vitamins provided) may be deduced as follows: (1) nitrogen balance was increased by lysine, not by tryptophan, therefore, lysine was the first limiting amino acid; (2) nitrogen balance with the subjects receiving maize plus lysine was improved by tryptophan, therefore, tryptophan was the second limiting amino acid; (3) nitrogen balance with the subjects receiving maize plus lysine and tryptophan was increased by adding isoleucine, but not by the addition of methionine, therefore isoleucine was the third limiting amino acid; (4) improvement with the first two or three limiting amino acids appeared comparable to the effect of a generous supplement of casein, it therefore seems unlikely that there is a serious deficiency of any other amino acid in maize, but we have not tried threonine or valine.

These conclusions conform very closely to the recent findings of Scrimshaw et al.[37,38] in children. They also found that lysine and tryptophan were the first two limiting amino acids in maize and that improvement of nitrogen re-

tention followed further additions of isoleucine maize thus supplemented gave nitrogen balances comparable to those obtained on milk protein. The addition of methionine to maize plus lysine and tryptophan depressed nitrogen balance. The I.N.C.A.P.[37,38] group went on to test the addition of threonine to a supplement of lysine plus tryptophan plus isoleucine in one child. It did not appear to be beneficial.

Our results differ in only one important detail. Scrimshaw's group found an increased nitrogen balance in three of four tests with tryptophan and little change in the fourth. Lysine alone was tried once and also produced a small improvement. In the Guatemala trials a multivitamin preparation was given throughout and the basal diet consisted of corn masa with some corn gluten added to increase the protein intake. The lysine:tryptophan ratio of this diet was 9.8:1 compared with 4.8:1 in the maize we used. It would seem that either lysine or tryptophan may be the first limiting amino acid in maize, depending on their relative concentrations.

For this reason we cannot recommend (a source of) lysine alone as a practical supplement to maize. There is a second reason why the minimal practical supplement of maize must provide both tryptophan and lysine. In the field, maize diets are likely to be deficient in available niacin. When we tested lysine as a supplement without extra vitamins in the diet, its benefit appeared to be much smaller. This result suggests a tryptophan-sparing action of niacin. It is, however, possible that removal of the other vitamins played a part, for example pyridoxine.

SUMMARY

The effect of supplementing the protein of whole maize meal was studied by prolonged nitrogen balance studies on eight metabolically normal men accustomed to eating large amounts of maize. In each experiment the subject served as his own control while different combinations of amino acids were given to replace a small part of the maize protein. It was found possible to measure the effects of these supplements under the natural conditions of a protein intake near 1 gm./kg. body weight (9 per cent dietary calories from protein) and under static conditions. The small positive nitrogen balance obtained with the subjects receiving unsupplemented maize under these conditions is considered to have been apparent and not real, because nitrogen losses via the skin were not measured. The data indicate that the chief limiting amino acids in the maize that was tested were lysine, tryptophan and isoleucine in that order of importance. However, lysine had little effect when extra vitamins were not given and other varieties of maize contain less tryptophan. A practical supplement of maize protein should therefore provide both tryptophan and lysine. Further benefit is to be expected from the inclusion of isoleucine.

ACKNOWLEDGMENT

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Relationships Between Animal Protein, Total Protein and Total Caloric Intakes in the Diets of Children from One to Eighteen Years of Age

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THIS paper is the third in a series dealing with the nutrient intake data of a group of children followed individually throughout the major portion of their growth period. These 125 children, sixty-four boys and sixty-one girls, whose dietary intakes of total calories, total protein and total animal protein have already been reported both cross-sectionally and in terms of individual longitudinal patterns[1,2] from one to eighteen years of age, belong to the Maturity Series of the Longitudinal Studies of Child Health and Development conducted since 1930 by the Department of Maternal and Child Health, Harvard School of Public Health.[3] In order to investigate further the sequential changes in the food intakes of children as individuals during their years of growth and development, a study has been made of the percentage of total protein derived from animal sources, and the percentage of caloric intake derived from total protein in a manner similar to that used in the previous papers.[1,2]

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This paper is No. 9 of the monodisciplinary reports in Series II of the Longitudinal Studies of Child Health and Development, Department of Maternal and Child Health, Harvard School of Public Health, Boston, Massachusetts.

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PERCENTAGE OF TOTAL PROTEIN FROM ANIMAL SOURCES

The percentage of protein derived from animal sources in the diets of these children offers a practical measurable estimate of the quality of protein eaten in any given age period. Thus, not only the absolute quantity of animal protein, but also the proportion which this forms to the total protein intake has been computed. A summary of the relationship of animal protein to total protein intake in this group of children is given in Table I. The percentages in this table are the mean values of animal protein given in Table I of the preceding paper[2] divided by the corresponding age and sex mean for total protein intake as given in Table I of a previous publication.[1]

These ratios of the means for each sex and at each yearly interval from one to eighteen years represent an average intake of animal protein which is approximately two-thirds or more of the total protein consumed for the same sex and age period. There is relatively little variation in these percentages based on average levels of intake. The lowest is 64.5 per cent of protein from animal sources among thirteen-year old boys, while the highest is 72.6 per cent among four year old girls.

TABLE I
Percentage of Total Protein Derived from Animal Sources by Age and Sex

Age (yr.)	Boys	Girls
1–2	70.2	72.5
2–3	70.5	72.3
3–4	71.4	72.1
4–5	70.9	72.6
5–6	69.0	72.3
6–7	69.2	71.3
7–8	68.6	70.4
8–9	67.3	70.3
9–10	68.8	69.4
10–11	68.6	69.2
11–12	66.3	67.3
12–13	65.6	64.6
13–14	64.5	66.3
14–15	65.2	67.2
15–16	66.0	69.7
16–17	68.1	69.8
17–18	68.7	70.0

Within this narrow range, the percentages for girls are slightly higher than those for boys at all ages except age twelve. In both sexes there is a tendency for higher percentages of animal protein to occur at the younger and older ages, with a minimum at twelve to thirteen for girls and thirteen to fourteen for boys. This age pattern is slightly more marked in girls than in boys.

Our figures for per cent of protein from animal sources, while similar to those of Widdowson[4] in age trends, are consistently higher than those for the British children. This is especially true of the early years except for the high figure of 76 per cent for one year old British girls. Only at age two were the British boys eating over 70 per cent of protein from animal sources, and at three ages twelve, thirteen and seventeen years the girls' intakes of animal protein were below 60 per cent of their total protein intake. The British data did not show a consistent difference between the sexes. The longitudinality of our material may explain this difference, as Widdowson's data from year to year is on different children at each age period.

CLASSIFICATION OF CHILDREN ACCORDING TO LONGITUDINAL PATTERNS OF PER CENT OF TOTAL PROTEIN FROM ANIMAL SOURCES

In order to obtain a more complete picture of individual variation in the source of protein, patterns of the percentage of total protein from animal sources were identified in a manner similar to that used previously in defining patterns of intake for total animal protein, total protein and total calories.[1,2] For each child the cumulative animal protein intakes during the three age periods one to six, six to twelve and twelve to eighteen, and during the entire age span from one to eighteen years were divided by the corresponding cumulative intake of total protein from animal sources. Percentages in the highest and lowest quartiles in each age period were classified as high (H) and low (L), respectively, while the middle two quartiles were designated as medium (M). The values defining these quartiles as well as the extreme values are given in Table II. The lowest and highest values observed during any of these age periods for any child were 50.9 per cent for one boy between twelve and eighteen years of age and 86.4 per cent for a girl between one and six years.

Table III shows the distribution of boys and

TABLE II
Percentages of Total Protein Derived from Animal Sources Which Define the Extreme Quartiles by Sex and Age Intervals

Age (yr.)	Boys				Girls			
	Lowest Value	25th Percentile	75th Percentile	Highest Value	Lowest Value	25th Percentile	75th Percentile	Highest Value
1–6	53.2	67.5	73.8	78.5	63.5	69.0	75.4	86.4
6–12	55.8	64.6	71.7	77.8	53.1	66.6	72.5	78.5
12–18	50.9	62.2	69.8	79.7	52.8	64.2	71.1	81.5
1–18	57.6	64.9	71.2	75.4	57.7	66.8	71.7	78.0

TABLE III

Frequency of Occurrence of Individual Longitudinal Patterns of Percentage of Total Protein Derived from Animal Sources

Patterns	No. of Boys	No. of Girls
HHH	8	6
MMM	9	8
LLL	3	4
MLL, HLL, HMM	(3 + 0 + 4) = 7	(1 + 1 + 2) = 4
LMM, LHH, MHH	(4 + 0 + 2) = 6	(7 + 0 + 2) = 9
MML, HHL, HHM	(7 + 0 + 2) = 9	(8 + 0 + 4) = 12
LLM, LLH, MMH	(7 + 0 + 4) = 11	(3 + 1 + 4) = 8
LML, LHL, LHM, MHL, MHM	(1 + 0 + 0 + 1 + 3) = 5	(0 + 0 + 0 + 0 + 3) = 3
MLM, MLH, HLM, HLH, HMH	(3 + 0 + 0 + 0 + 1) = 4	(4 + 1 + 0 + 0 + 1) = 6
LMH	1	0
HML	1	1

NOTE: H = High; M = Medium; L = Low.

girls according to the various longitudinal patterns. The first three patterns in this table (HHH, MMM and LLL), imply relatively consistent percentages of animal protein throughout the three age periods one to six, six to twelve and twelve to eighteen. The next four lines refer to patterns with either descending or ascending trends between two of the age periods. The eighth and ninth lines contain patterns characterized by increase, followed by decrease, or the reverse. The last two (LMH and HML) describe continuous increase or continuous decrease from period to period. It is interesting to note that as in the previous dietary pattern distributions, patterns involving extreme shifts between high and low ratings are very rare, whereas patterns involving consistent ratings in successive age periods are relatively common. Only one boy and three girls were found with any of the following patterns: HLL, LHH, HHL, LLH, LHL, LHM, MHL, MLH, HLM and HLH. If the combinations of ratings in successive age periods occurred by chance these patterns should have occurred in sixteen boys and fifteen girls. On the other hand, the pattern HHH which should have occurred by chance in about one boy and one girl was observed in eight boys and six girls.

Some of the more extreme types of variation between children in the pattern of percentage of total protein intake derived from animal sources are shown in Figure 1. In each of the four sections of this figure two strikingly different cases are shown in comparison with the mean value for the appropriate sex. In the upper two sections the contrast is between children rated as having consistently high and consistently low intakes of animal protein. The lower left section shows two boys, both of whom obtained about 68 per cent of their protein from animal sources during the age period from twelve to eighteen, but whose previous intakes were strikingly different, particularly from about age four to age ten. Still another contrast is shown by the two girls in the lower right section. One (Case 159) started with relatively low percentages of animal protein which increased markedly between six and twelve years of age, while the other (Case 217) followed a reverse type of pattern with an abrupt decline in percentage of animal protein between the ages of nine and twelve years.

PERCENTAGE OF TOTAL CALORIES OBTAINED FROM TOTAL PROTEIN

Consideration of protein nutrition also involves the adequacy of the caloric intake in conjunction with the quantity and quality of the protein intake. Over the years it has been common practice to express the needs of the growing child for protein as a percentage of his total daily caloric needs. It is, therefore, of interest to determine not only the proportion of total protein derived from animal

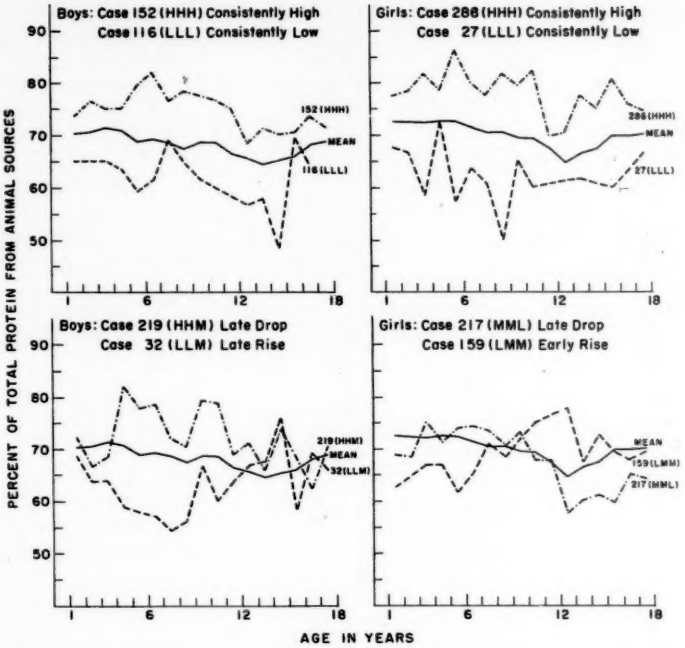
Boys: Case 152 (HHH) Consistently High
Case 116 (LLL) Consistently Low
Girls: Case 286 (HHH) Consistently High
Case 27 (LLL) Consistently Low
Boys: Case 219 (HHM) Late Drop
Case 32 (LLM) Late Rise
Girls: Case 217 (MML) Late Drop
Case 159 (LMM) Early Rise
PERCENT OF TOTAL PROTEIN FROM ANIMAL SOURCES
AGE IN YEARS
MEAN

FIG. 1. Selected individual curves of the percentage of total protein derived from animal sources.

TABLE IV

Percentage of Total Calories Derived from Total Protein by Age and Sex

Age (yr.)	Boys	Girls
1–2	13.5	13.9
2–3	13.1	13.7
3–4	13.0	13.2
4–5	13.1	13.3
5–6	12.9	13.3
6–7	12.9	13.1
7–8	13.0	13.2
8–9	12.9	12.9
9–10	13.0	13.1
10–11	13.0	13.0
11–12	12.6	12.5
12–13	12.2	12.4
13–14	12.4	12.7
14–15	12.2	12.5
15–16	12.3	12.7
16–17	12.5	12.8
17–18	12.5	12.8

sources, but also the percentage of total calories derived from total protein.

Table IV gives by age and sex the percentage of total calories derived from the protein in the diet of these children. It should be remembered that these figures are computed from the means and do not indicate the rather striking individual variabilities which exist within this group of 125 children followed longitudinally from one to eighteen years.

Table V gives the lowest and highest percentages of total calories derived from protein for each sex and in each age interval, one to six, six to twelve, twelve to eighteen and one to eighteen, and also shows the values defining the quartiles. While it can be said that the majority of the children at all ages had protein intakes represented by 12 to 14 per cent of their caloric intakes, there is a wide variation around this middle zone. The values range as high as 16.1 per cent for a girl in the age range twelve to eighteen, and as low as 9.7 per cent for a boy in the age range six to twelve.

Table VI shows the distribution of the sixty-four boys and sixty-one girls according to their various longitudinal patterns and pattern groups representing the percentage of

TABLE V

Percentages of Total Calories Derived from Total Protein Which Define the Extreme Quartiles by Sex and Age Intervals

Age (yr.)	Boys				Girls			
	Lowest Value	25th Percentile	75th Percentile	Highest Value	Lowest Value	25th Percentile	75th Percentile	Highest Value
1–6	10.5	12.4	13.7	15.1	11.3	12.7	14.2	15.7
6–12	9.7	12.2	13.4	15.6	10.3	12.2	13.6	15.5
12–18	10.1	11.8	13.0	14.9	10.1	12.0	13.3	16.1
1–18	10.7	12.3	13.1	15.0	10.7	12.3	13.8	15.0

TABLE VI

Frequency of Occurrence of Individual Longitudinal Patterns of Percentage of Total Calories Derived from Total Protein

Patterns	No. of Boys	No. of Girls
HHH	4	4
MMM	9	12
LLL	4	4
MLL, HLL, HMM	(2 + 0 + 2) = 4	(1 + 0 + 1) = 2
LMM, LHH, MHH	(5 + 1 + 2) = 8	(3 + 0 + 5) = 8
MML, HHL, HHM	(5 + 1 + 4) = 10	(4 + 0 + 5) = 9
LLM, LLH, MMH	(3 + 0 + 5) = 8	(2 + 0 + 1) = 3
LML, LHL, LHM, MHL, MHM	(2 + 0 + 1 + 0 + 3) = 6	(4 + 0 + 0 + 0 + 1) = 5
MLM, MLH, HLM, HLH, HMH	(4 + 2 + 1 + 0 + 2) = 9	(6 + 1 + 1 + 0 + 2) = 10
LMH	0	2
HML	2	2

total calories derived from total protein in the various age groups one to six, six to twelve and twelve to eighteen. Values in the highest quartile are designated as high (H), those in the two middle quartiles as medium (M), and those in the lowest quartile as low (L).

COMMENTS

Consideration of protein nutrition involves consideration of the adequacy of caloric intake as well as the quality and quantity of protein consumed. This is especially true in young children when a deficiency of only 100 calories may result in 25 gm. of total protein intake needed for growth being burned for energy. Macy[5] has stated "that a difference of 10 cal. per kilogram of body weight may spell success or failure in making satisfactory progress in visible and invisible growth."

SUMMARY

The percentage of total protein derived from animal sources in the diets of children is presented and is followed by the percentage of total calories derived from total protein. These data have been compiled for each of 125 children at each age interval from one to eighteen years.

In both sexes there is a tendency for higher percentages of animal protein to occur at the younger and older ages, with a minimum at twelve to thirteen years for girls, and thirteen to fourteen years for boys. These ratios of the means for each sex and at yearly intervals represent an average percentage of animal protein which is approximately two-thirds or more of the total protein consumed. The average values for the girls for the first nine years are somewhat higher than those of the boys.

The majority of these boys and girls at all ages had protein intakes representing approximately 12 to 14 per cent of their total caloric intakes.

A more complete picture of the individual variations in the percentage of animal protein consumed is obtained from a study of the longitudinal patterns of the percentage of total protein from animal sources at certain specified age intervals, i.e., one to six, six to twelve, twelve to eighteen and one to eighteen years.

The lowest and highest percentage of calories derived from total protein were 9.7 per cent and the highest 16.1 per cent, respectively. Data are presented showing the distribution of these boys and girls according to their longitudinal patterns of percentage of total calories derived from protein, and allow consideration of their variability from one age period to another.

Finally, with the completion of this report, we have comparable nutrient intake material over a seventeen-year span on these 125 children in five categories: (1) total caloric intakes; (2) total protein intakes; (3) total animal protein intakes; (4) percentages of animal protein from total protein; and (5) percentage of total calories from total protein.

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Studies on Fat Metabolism in Kwashiorkor

II. Serum Polyunsaturated Fatty Acids

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As early as 1930[1] the so-called "essential fatty acids" were shown in the rat to be necessary for maximal growth and nutrition of the skin. More recently these polyunsaturated fatty acids have been reported to be associated with several other functions: maintenance of subcutaneous capillary pressure,[2,3] successful pregnancy and lactation[4] as well as protection against respiratory infections and x-ray radiation.[5] It has also been reported that deficiency of these acids is associated with diarrhoea, skin lesions and perianal irritation in newborn infants.[6,7]

There is some resemblance between these alleged manifestations of fatty acid deficiency and the symptomatology of kwashiorkor (infant protein malnutrition). Although it is generally accepted that kwashiorkor is the result of protein deficiency, there is no doubt that the diets of those in whom it develops are also frequently low in fat content. Further, the well documented observation that the liver is usually fatty in subjects with kwashiorkor indicates a defect in fat metabolism which needs investigation. Earlier work in this laboratory[8] has confirmed reports that total serum cholesterol concentrations in those with kwashiorkor are low on admission and that they rise to control levels after a transient peak. If one accepts the theory that cholesterol is part of the soluble lipoprotein complex, one is tempted to suggest that the concentration of serum cholesterol (its ester or the fatty acids of its ester) might be a reflection of fat transport.

Brown and Hansen[9] have reported significantly lower concentrations of serum linoleic and arachidonic acids in infants with eczema and frequent rises of linoleic acid levels on treatment with unsaturated vegetable oils. These investigators[10,11] have also observed significantly lower levels of di-, tetra- and hexaenoic acids in the serum of thirty-four malnourished infants as compared with sixty well nourished subjects. The total fatty acids in the two groups were similar and the trienoic acid concentration was significantly elevated in the malnourished state. For these reasons, fractionation of the total serum polyunsaturated fatty acids was undertaken in these subjects on admission and during various stages of recovery from kwashiorkor.

CLINICAL MATERIAL AND METHODS

Total serum fatty acids and five polyunsaturated fatty acids were estimated in nineteen patients with kwashiorkor with a mean age of one year (range one to three years) and in eight well nourished (convalescent poliomyelitis) infants with a mean age of two years and three months (range ten months to three and a half years). The subjects with kwashiorkor used in this study were non-White males typical of those seen in Cape Town and were selected on the basis of criteria previously described.[12] The dietary protein of these infants had all been extremely poor and diarrhoea was the most common accompanying symptom at the time of admission. We have defined our criteria of response in greater detail elsewhere.[12,13] In brief, cure is said to be "initiated" when within ten to twenty-one days the downward course of the disease is reversed,

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TABLE I

Effect of Dietary Fat on Serum Fatty Acid Concentrations of Infants with Kwashiorkor

	Per Cent Total Fatty Acids				Plasma Concentration (mg./100 ml.)			
	Fat-Free Synthetic Amino Acids	Skim Milk	Half-Cream Milk	Mixed Diet	Fat-Free Synthetic Amino Acids	Skim Milk	Half-Cream Milk	Mixed Diet
Admission to Tenth Day of Treatment								
Total fatty acids					−2.10	−1.75	0.49	3.46
					1.33	1.02	2.32	...
					0.99	...	−0.86	...
					0.45	...	−1.42	...
Mean					−0.17	−0.36	−0.13	3.46
Monoethenoic plus saturated fatty acids	0.16	8.72	4.71	6.32	−1.78	−1.06	0.58	3.46
	2.07	0.87	0.07	...	1.05	0.86	−0.58	...
	1.99	...	−0.33	...	0.44	...	−1.19	...
Mean	1.41	4.80	1.49	6.32	−0.09	−0.10	−0.40	3.46
Dienoic fatty acids	−2.49	−8.10	−2.09	−3.75	−33.38	−58.75	−1.40	1.22
	−2.16	0.18	1.50	...	−8.71	11.39	1.43	...
	0.07	...	0.12	...	7.34	...	−14.55	...
Mean	−1.53	−3.97	−0.16	−3.75	−11.58	−23.68	−4.84	1.22
Trienoic fatty acids	1.63	0.18	0.18	0.49	5.90	−0.61	0.94	6.16
	0.27	0.12	1.13	...	3.03	1.50	4.80	...
	0.30	...	0.60	...	2.18	...	0.41	...
Mean	0.74	0.15	0.64	0.49	3.70	0.45	2.05	6.16
Tetraenoic fatty acids	0.92	−1.20	−1.98	−1.54	−0.19	−8.77	−5.56	−3.89
	−0.18	−1.41	−1.95	...	−0.16	−1.38	−10.98	...
	−0.48	...	−0.77	...	−0.82	...	−7.72	...
Mean	0.09	−1.31	−1.57	−1.54	−0.39	−5.08	−8.09	−3.89
Pentaenoic fatty acids	0.27	−0.24	−0.36	−0.50	0.15	−2.09	−0.99	−1.38
	−0.09	−0.04	−0.10	...	−0.41	0.65	−0.79	...
	−0.60	...	0.03	...	−2.55	...	−0.53	...
Mean	−0.14	−0.14	−0.14	−0.50	−0.94	−0.72	−0.77	−1.38
Hexaenoic fatty acids	−0.41	0.73	−0.77	−1.02	−5.00	1.76	−2.17	−2.33
	−0.20	0.13	−0.66	...	−0.81	2.24	−3.80	...
	−1.23	...	0.42	...	−4.80	...	0.03	...
Mean	−0.61	0.43	−0.33	−1.02	−3.53	2.00	−1.98	−2.33
Tenth Day of Treatment to Cure								
Total fatty acids							0.25	0.03
							−1.10	−1.29
							...	−1.24
							...	−1.43
							...	3.93
							...	−1.15
Mean							−0.43	−0.19

TABLE I (*Continued*)
Effect of Dietary Fat on Serum Fatty Acid Concentrations of Infants with Kwashiorkor

	Per Cent Total Fatty Acids				Plasma Concentration (mg./100 ml.)			
	Fat-Free Synthetic Amino Acids	Skim Milk	Half-Cream Milk	Mixed Diet	Fat-Free Synthetic Amino Acids	Skim Milk	Half-Cream Milk	Mixed Diet
Tenth Day of Treatment to Cure (Continued)								
Monoethenoic plus saturated fatty acids			−2.89	−0.23			−1.32	0.01
			−8.76	1.09			...	−1.04
			...	1.15			...	3.89
			...	8.19				
			...	−1.98				
Mean			−5.82	1.65			−1.32	0.95
Dienoic fatty acids			2.07	−1.85			8.91	−6.51
			6.92	1.60			19.54	−3.63
			...	−4.95			...	−32.95
			...	−5.13			...	3.10
			...	0.83			...	−3.28
Mean			4.50	−1.90			14.22	−8.65
Trienoic fatty acids			0.64	0.98			2.19	3.54
			0.81	−0.96			1.59	−6.76
			...	1.13			...	2.70
			...	0.29			...	6.83
			...	0.01			...	−3.14
Mean			0.73	0.29			1.90	0.63
Tetraenoic fatty acids			0.56	0.89			2.14	3.50
			1.53	−0.73			4.64	−6.03
			...	0.76			...	−0.71
			...	−1.38			...	...
			...	...			...	−0.77
			...	0.11			...	−1.88
Mean			1.05	−0.07			3.39	−1.18
Pentaenoic fatty acids			−0.25	0.22			−0.59	0.82
			0.12	−0.51			0.07	−2.93
			...	−0.20			...	−2.34
			...	−0.50			...	−1.28
			...	0.25			...	0.55
Mean			−0.06	−0.15			−0.26	−1.04
Hexaenoic fatty acids			−0.69	0.17			−1.63	0.62
			−0.63	−0.42			−4.03	−3.12
			...	−1.49			...	3.41
			...	−1.44			...	−3.82
			...	0.61			...	1.29
Mean			−0.66	−0.51			−2.83	−0.33

the skin lesions are healed or are healing, the edema is gone and there is definite improvement in attentiveness and appetite. Serum albumin concentration must have risen to concentrations greater than 3 gm./100 ml.

The infants all received an electrolyte solution

TABLE
Serum Fatty Acids of Control

Subjects	No.*	Total Serum Cholesterol (mg./100 ml.)	Total Fatty Acids (mg./100 ml.)	Saturated + Monoethenoic Acids†		Dienoic Acids	
				T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.
Control subjects‡	8	171 ± 35§	407 ± 12	84.5 ± 3.9	336 ± 10	8.6 ± 2.7	33.7 ± 12.9
Infants with kwashiorkor on admission to hospital	15	151 ± 49 $p < 0.02$ ‖	385 ± 17	83.0 ± 3.4	362 ± 14	10.3 ± 4.2	42.7 ± 17.8
Infants with kwashiorkor after 10 days of treatment	12	182 ± 45	484 ± 12	84.8 ± 4.1	416 ± 12	10.0 ± 3.4	48.0 ± 19.5
Infants with kwashiorkor at initiation of cure	11	188 ± 51	438 ± 15	86 4 ± 3.3	382 ± 14	8.1 ± 2.5	34.0 ± 11.0
Infants with kwashiorkor on admission who subsequently died¶	6	...	338 ± 11	82.0 ± 6.6	288 ± 10	10.3 ± 5.3	34 3 ± 15.7

NOTE: T.F.A. = Total fatty acids.
* Since the subjects were not always studied through to initiation of cure, the number of cases varies.
† The saturated plus monoethenoic acids were calculated as the difference between the total fatty acids and the sum of the polyenoic acids.

with glucose for the first twelve to twenty-four hours after admission. They were then offered various low-fat formulas (skim milk or fat-free synthetic formula[14]) for two weeks and finally a mixed diet (Table 1). The mineral and vitamin supplements were identical in all the synthetic formulas and based on that found in milk. Fasting (six hours) blood samples were taken by venipuncture at the time of admission, ten days after admission to approximate the average period of maximum serum cholesterol concentration[8] and when cure was judged to be initiated.

Serum lipids were extracted in triplicate, with slight modification, according to the procedure of O'Connel et al.[15] The total fatty acids were determined by weight in tared isomerisation tubes prior to conjugation. The unsaturated fatty acids were estimated by the alkali isomerisation method[16,17] using a fifteen minute conjugation interval and the extinction coefficients of Hammond and Lundberg.[17] We were able to check these coefficients for linoleic, linolenic and arachidonic acids and found them in close agreement. The pentaenoic acids present were assumed to be the C_{20} variety.[17] Data for monoethenoic acid (i.e., oleic) plus the saturated fatty acids were calculated by difference.

Total serum cholesterol was estimated in duplicate using the method of Abell et al.[18] as modified by Anderson and Keys.[19]

RESULTS

The mean concentration of total, saturated and polyunsaturated fatty acids in the serum of eighteen infants with kwashiorkor and eight control infants are tabulated in Table II and illustrated in Figures 1 and 2. It has been suggested that such data be expressed both in absolute amounts (mg./100 ml. serum) and as percentage of total fatty acids. The concentration of total cholesterol in the serum of these subjects is also tabulated in Table II. These serum lipid concentrations estimated at three stages in the treatment of infants with kwashiorkor, were compared with the control figures using Student's "t" test for comparing means.

II
Subjects and Infants with Kwashiorkor

Trienoic Acids		Tetraenoic Acids		Pentaenoic Acids		Hexaenoic Acids	
T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.
1.6 ± 0.5	5.9 ± 1.4	2.6 ± 1.7	9.7 ± 6.6	0.7 ± 0.2	2.7 ± 1.4	2.1 ± 0.9	8.1 ± 3.1
0.9 ± 0.4 $p < 0.01$	3.9 ± 1.9 $p < 0.05$	2.9 ± 1.2	11.5 ± 3.9	0.8 ± 0.1	3.3 ± 1.5	1.8 ± 0.7	7.5 ± 2.3
1.5 ± 0.7	7.3 ± 3.6	2.1 ± 1.0	9.1 ± 5.1	0.6 ± 0.3	2.6 ± 0.9	1.2 ± 0.8 $p < 0.05$	4.7 ± 2.5 $p < 0.05$
1.9 ± 0.5	7.9 ± 2.7	2.2 ± 0.8	9.0 ± 3.2	0.5 ± 0.2	1.9 ± 0.8	1.0 ± 0.5 $p < 0.01$	4.2 ± 2.1 $p < 0.01$
1.0 ± 0.4 $p < 0.05$	3.0 ± 0.9 $p < 0.01$	3.0 ± 1.5	10.1 ± 4.6	0.9 ± 0.3	3.4 ± 1.7	2.1 ± 0.3	7.5 ± 2.6

‡ Convalescent poliomyelitis infants of a similar age receiving a mixed hospital diet served as control subjects.
§ Mean ± standard deviation.
|| Serum lipids of the infants with kwashiorkor were compared with those of the control subjects using Student's "t" test for comparing means.
¶ The deaths all occurred within the first nine days of treatment.

On admission to the hospital, the trienoic fatty acids in the serum of infants with kwashiorkor, were significantly less than the control series when expressed as mean concentration ($p < 0.01$) or percentage of fatty acids ($p < 0.05$). This difference was also observed in those infants who subsequently died ($p < 0.05$ and $p < 0.01$, respectively). The mean hexaenoic fatty acid concentration or percentage fell significantly below the control figure after ten days of treatment ($p < 0.05$) and after cure was initiated ($p < 0.01$). The concentration of total cholesterol in the serum was significantly lower ($p < 0.02$) than the control series and increased with treatment to a mean concentration which was not different from the control mean.

There also appeared to be a general tendency for the concentrations or percentages of total fatty acids, monoethenoic plus saturated fatty acids and trienoic fatty acid to increase during the first ten days of treatment and then to plateau or decrease as cure was initiated. The concentration or percentage of the remaining fatty acids (di-, tetra- and pentaenoic acid) tended to decrease throughout treatment.

Since it would appear that each of the infants with kwashiorkor has rather unique unsaturated fatty acid concentrations we also considered these experiments as internally controlled and analysed the changes in lipid pattern using the t test for comparing mean differences (Table III). We have plotted these mean changes in serum concentration for all the fatty acids and total cholesterol in Figure 3.

During the first ten days of treatment the concentration of trienoic acid increased ($p < 0.02$) whereas the concentration of hexaenoic acid decreased ($p < 0.05$). Similarly, comparisons of these data, expressed as per cent of the total fatty acids, indicate that the hexaenoic acid percentage decreased during the

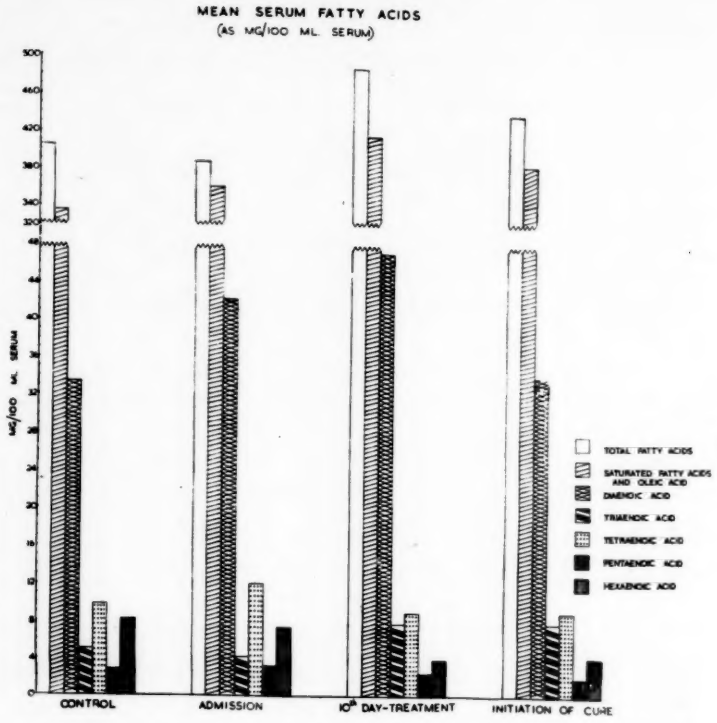

FIG. 1. Mean serum fatty acids (as serum concentration mg./100 ml.) of control subjects and infants with kwashiorkor. For the standard deviation and number of infants comprising each group see Table I.

first ten days of treatment ($p < 0.05$) and as cure was initiated ($p < 0.05$).

General trends in the changes of the other lipid constituents were observed to be similar

TABLE III

Changes in Serum Fatty Acid Concentration During Treatment of Kwashiorkor

Period of Treatment	No.*	Total Serum Cholesterol (mg./100 ml.)	Total Fatty Acids (mg./100 ml.)	Saturated + Monoethenoic Acids†		Dienoic Acids	
				T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.
Admission to "10 days"	7	22	18.86	1.65	9.5	−0.83	−3.89
"10 days" to "cure"	9	6	−5.67	1.72	−18.6	−1.93	−13.46
Admission to "cure"	5	44	106	4.9	84.0	−3.26	−10.14

NOTE: T.F.A. = Total fatty acids.

* Since the subjects were not always studied through to initiation of cure the number of cases varies.

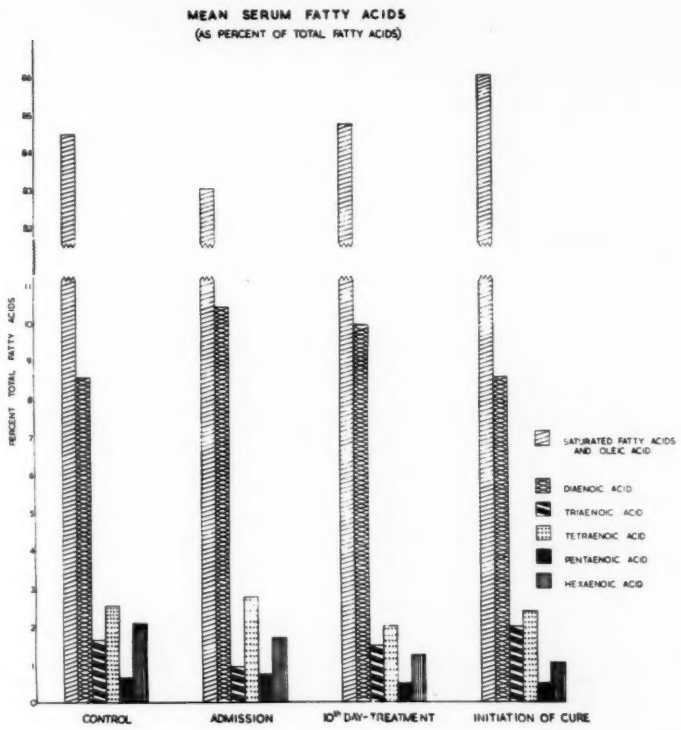

FIG. 2. Mean serum fatty acids (as per cent of total fatty acids) of controls and infants with kwashiorkor. For the standard deviations and number of infants comprising each group see Table I.

TABLE III (*Continued*)

Trienoic Acids		Tetraenoic Acids		Pentaenoic Acids		Hexaenoic Acids	
T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.	T.F.A. (%)	mg./100 ml.
0.61	3.06 $p < 0.02$‡	−0.85	−3.18	−0.15	−0.66	−0.52 $p < 0.05$	−2.76 $p < 0.05$
0.36	0.65	−0.22	−2.53	−0.15	−1.05	−0.26	−0.18
0.75	3.98	−1.23	−3.48	−0.31	−1.19	−0.91 $p < 0.05$	−3.75

† The saturated plus monoethenoic acids were calculated as the difference between the total fatty acids and the sum of the polyenoic acids.

‡ The mean change of the serum lipids of the subjects was tested statistically using Student's "t" test.

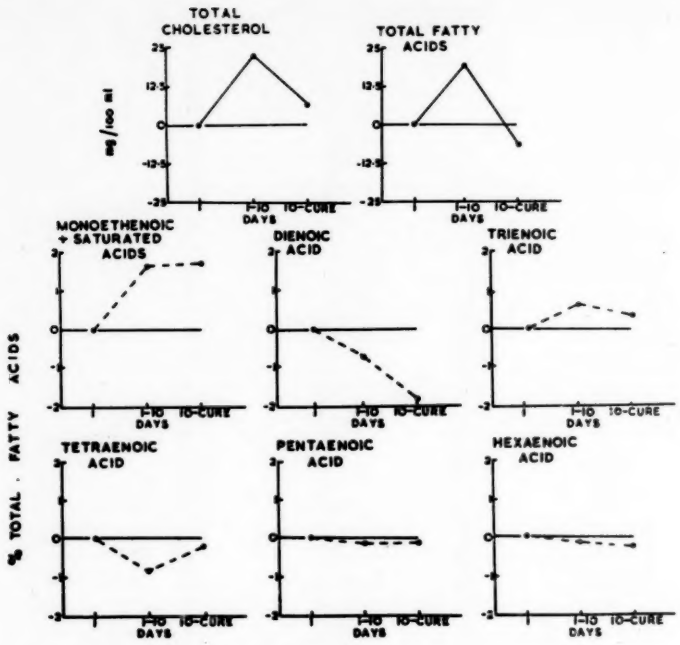

FIG. 3. Mean changes in serum fatty acids and cholesterol during the treatment of infants with kwashiorkor. See Table II for the statistical conclusions.

to the ones described for the mean data.

COMMENTS

We have previously reported a significant rise in concentration of cholesterol during recovery from kwashiorkor. This rise appeared to be maximal during the first two weeks of adequate treatment. An arrested rise or fall was found to be associated with the onset of complications or inadequate therapy. Others[20,21] have also observed an increase in the serum concentration of cholesterol in infants with kwashiorkor treated with skim milk.

Serum cholesterol, or more precisely the unsaturated fatty acid ester of cholesterol, has been thought to be essential for the transport of fat. Concentrations of plasma cholesterol have been reported to fall during the accumulation of fat in the livers of rats receiving fat-free diets.[22] It is possible therefore, that serum concentrations of cholesterol or its esters may reflect the rate at which liver fat is cleared during recovery from kwashiorkor.

The range of serum polyunsaturated fatty acid concentrations found in this series and elsewhere[23,24] is considerable. This may prove to be a normal finding in that the pattern of unsaturated fatty acid concentration is extremely variable or unique for each subject. Differences of severity of illness on admission and of dietary fat intakes in the home may also account for some of the variability. Preliminary reports[25] suggest that serum levels of di-, tri- and tetraenoic fatty acids reflect the dietary intake of linoleic acid. It has also been shown in several species that serum linolenate concentration is reduced by the ingestion of polyunsaturated fatty acids[26] and increased following the ingestion of a fat-free diet.[27,28]

In order to describe the polyunsaturated fatty acid concentrations of these infants with kwashiorkor as abnormal, they would have to be compared with data from a group of healthy infants ingesting a similar type and amount of fat. Unfortunately, we do not have adequate information about the fat intake of these in-

fants with kwashiorkor in their home environment. It is of interest, however, that the serum fatty acid concentrations of our infants with kwashiorkor at the time of admission, including the six who died, are similar to those of the well nourished infants eating a mixed hospital diet which we have used as controls.

These observations are somewhat in conflict with the work of others who report evidence of polyunsaturated fatty acid deficiency in poorly nourished infants[11] and in adults on a low fat diet.[29] However during the recovery of our infants with kwashiorkor a trend in serum fatty acid concentrations became apparent which is similar to that which occurs during the development of the essential fatty acid deficiency syndrome in the rat. In rats,[30] which have received completely fat-free diets for a considerable period of time and in which certain characteristic symptoms develop, the serum concentrations of trienoic fatty acid increases while the concentration of the di-, tetra- and hexaenoic fatty acids decreases. Our data would suggest that during the treatment of infants with kwashiorkor, pentaenoic acid also tends to fall with the di-, tetra- and hexaenoic fatty acids.

If one accepts the theory that unsaturated fatty acids are associated with cholesterol transport, then as Holman suggests[31] "The need to transport large amounts of one component of the lipid mixture requires the animal to synthesize or release from its tissues matching quantities of the other components . . . . If such a condition persists the tissues may become depleted of essential fatty acids and the composition of its plasma lipids may become abnormal." Such a situation might well occur during the recovery from kwashiorkor.

Polyunsaturated fatty acids are believed to be essential constituents of protoplasm and the rapid repletion of tissues in the infant with kwashiorkor must cause a further drain on the reserve of these acids.

Another possible explanation for the data is that several of these infants were called upon to metabolise an amount of saturated dietary fat (present in the skim milk) in addition to clearing the liver of fat. Although there are an insufficient number of infants on each diet (Table 1) there does not appear to be a consistent relationship between this small amount of dietary fat and the serum polyunsaturated fatty acid pattern which developed in these subjects recovering from kwashiorkor.

The rapidity with which a so-called "essential fatty acid deficiency pattern of serum lipids" becomes manifest in our cases is of interest. Presumably this suggests either (1) that the mobilisation of a large amount of hepatic and depot fat imposes a considerable transitory requirement on the constituents which together make up the soluble lipid complex, or (2) that the tissue reserves of the polyunsaturated fatty acids are minimal.

Serum concentrations of polyunsaturated fatty acids do not necessarily reflect the status of tissue reserves. If there is no shortage of these fatty acids, as might be suggested by the fact that the fat-free synthetic formulas[13] have been used with partial success in treating infants with this syndrome, then two possible mechanisms may be suggested to explain the etiology of the fatty liver. There may be a deficiency of one of the components of the soluble lipid complex (e.g., lipoprotein) or the liver is unable to combine these components in the required fashion (e.g., deficiency of an esterase required for the synthesis of unsaturated cholesterol esters).[8,32–35]

SUMMARY

Serum fatty acids were fractionated in eight control infants and in nineteen infants with kwashiorkor.

During the treatment of infants with this syndrome, profound changes in the serum pattern of various polyunsaturated fatty acids were observed. It is of interest that these changes mimic those which are reported to occur during the development of "essential fatty acid" deficiency in the rat. It is suggested that the underlying basis of these changes is the mobilisation of hepatic and depot fat during the recovery phase.

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On the Vitamin B Complex Content of the Hair of Normal and Pellagrous Subjects

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THE protective role played by most of the B-complex vitamins on the epidermis is known. We thought it would be of interest to investigate the content of such vitamins in the hair of pellagrous subjects. However, we had no data on the hair content of normal subjects. Therefore, we had to determine the values in control subjects immediately after cutting their hair and after several months of conservation. This second determination was indispensable for interpreting the values found in pellagrous subjects, which were determined several months after taking the sample.

The B-complex vitamins quantitatively determined were niacin, riboflavin, pyridoxine, and pantothenic acid. The determinations were made at the Foch Research Center in Paris, under the supervision of Mlle Marnay.

NIACIN

We used the method of Snell and Wright,[1] i.e., opacimetric measurement of the growth of Lactobacillus arabinosus. The fat is removed from finely cut hair by means of two or three applications of chloroform, with agitation for fifteen minutes each time. The sample is left in the dryer overnight, under vacuum. To a sample of 100 mg. of hair from which the fat has been removed are added 15 ml. N HCl; this solution is passed through the autoclave at 120°c. for sixty minutes and, after filtration, the pH is brought to 6.8 and the suitable dilutions prepared.

Content of Hair from Normal Subjects. We made twenty-five determinations on apparently healthy subjects between two and ninety-five years of age. No treatment likely to have included nicotinamide had been prescribed for them recently. Table I shows the results obtained, which ranged from 7 to 43 μg. of nicotinic acid/gram of hair, the average being 18 μg. Color, thickness and age did not appear to have any significant bearing on these data.

Content after Three Months of Storage. Quantitative determinations were carried out on nine samples a short time after cutting; these were then kept in locks at room temperature, in the dark, and tested a second time three months later.

The following results were obtained: before storage, 20, 18, 13, 7, 9, 18, 24, 7 and 12 μg./gm. of hair; after storage, 19, 16, 13, 8, 9, 18, 25, 7 and 14 μg/gm. of hair.

Storage did not result in any reduction in nicotinic acid content. The figures remain constant, and the initial average of 14 μg./gm. is found to be almost exactly the same after three months.

Content of Hair from Pellagrous Subjects. The samples were taken from subjects with pellagra, which was rampant in Yugoslavia and affected Albanian and Serbian subjects. The quantitative determination was performed three months after cutting. Table II gives the results obtained in the twenty-five determinations. The arithmetic average is 9 μg./gm. of hair.

This figure is therefore significantly lower as compared to that obtained under similar conditions for the hair of healthy French subjects. The hair of pellagrous subjects, therefore, actually seems to have a lower nicotinic acid

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TABLE I
Niacin Content of the Hair of Normal Subjects

Age (yr.)	Sex	Color	Texture	Niacin Content (μg./gm. of hair) (fat removed)
2	M	Blond	Very fine	31.5
6	F	Dark brown	Fine	9.7
11	M	Dark brown	Fine	13
16	F	Dark brown	Thick	13.5
24	M	Dark brown	Fine	14.1
26	F	Very dark brown	Rather fine	20.7
26	M	Black	Medium	24.3
26	F	Light brown	Medium	18.6
29	M	Dark brown	Thick	20.4
38	M	Brown	Rather fine	27
46	M	Brown (some white)	Rather fine	20
47	M	Black	Rather thick	24
49	F	Dark brown	Very fine	9.4
60	F	Dark brown (some white)	Rather fine	18.6
64	F	Very dark brown	Fine	18.7
74	F	Gray	Rather fine	9.2
74	F	Black (some white)	Medium	43
74	F	Brown	Very fine	7.3
75	F	Black (some white)	Medium	37.1
76	F	Light brownish blond (some white)	Rather fine	7.5
83	F	Dark brown (some white)	Fine	10.1
83	F	White	Fine	7.4
85	F	Dark brown and some white	Rather fine	17.7
85	F	Dark brown and some white	Very fine	16.8
92	F	White and light brown	Rather thick	12.4
Average				18

content. If the results are analyzed in detail, it is observed that there are normal or even high concentrations, but that the average remains appreciably lower than in normal subjects.

RIBOFLAVIN

We used the microbiologic method of Lactobacillus casei growth and quantitative determination of the lactic acid formed according to the technic of Snell and Strong.[2] The hair underwent the same preparation as for the preceding determinations. After drying under vacuum, a sample was placed in 0.1 N HCl and passed through the autoclave for twenty minutes at 120°c.

Content of Hair from Normal Subjects. In fifteen subjects the values are quite constant and range from 1 μg./gm. of hair, the lowest concentration, to 2.6 μg./gm. of hair, the highest concentration. The most prevalent concentrations lie between 1.2 and 2 μg./gm Neither the effect of thickness or pigmentation of the hair, nor that of the age of the subject, was found to be of any significance.

Content after five months of storage. The quantitative determination of riboflavin in seven samples kept in the dark for five months yielded the following results: before storage, 1.2, 2.5, 2.6, 2.2, 1.6, 2, and 2 μg./gm. of hair; after storage, 0.5, 1.2, 1.2, 0.9, 1.2, 0.2 and 0.8 μg./gm. of hair; the average being 2 μg./gm. of hair before storage and 0.8 μg./gm. of hair after storage.

Storage was therefore found to result in a general decrease in the second determination; this decrease is, on the average, 60 per cent of the initial concentration.

Content of Hair from Pellagrous Subjects. The hair was collected in July and August 1958 but could not be quantitatively determined in Paris until five months later. The nineteen determinations made are recorded in

TABLE II

Niacin Content of Hair from Pellagrous Subjects (After Three Months of Storage)

Age (yr.)	Sex	Color	Texture	Nicotinic Acid Content (μg./gm. of hair) (fat removed)
1	M	Golden blond	...	6.2
5	M	Light blond	Fine	8.4
8	M	Light brown	Medium	4.1
8	M	Golden blond	...	7.4
12	F	Auburn	...	7.3
12	M	Brown	Medium	6.2
16	M	Brown	Medium	8.9
17	M	Dark brown	...	14.5
20	F	Titian red	...	4.9
20	F	Auburn	Medium	5.4
24	F	Brown	Fine	8.9
30	F	Black	Thick	21.6
40	F	Brown	Fine	4.5
40	F	Auburn	Medium	8.8
40	F	Dark brown (some white)	Fine	12.1
45	F	Dark brown	...	6.2
45	F	Black	Medium	17.3
48	F	Dark brown (some white)	...	15.5
50	F	Black	Thick	13.2
50	F	Dark brown	Medium	19.3
60	F	White (some dark brown)	...	5.4
60	F	Auburn	Thick	5.4
60	F	Auburn and white	Fine	11.6
65	F	Brown	Medium	10.1
70	F	Golden brown	Medium	11.9
Average				9

Table III. It will be noted that certain figures are especially high, accounting for a higher general average than that determined in the control subjects after storage for the same length of time. Indeed, the average obtained in the pellagrous subjects was 1.5 μg./gm. If it is considered that this hair lost 60 per cent of its initial content during the five months following its cutting, we are led to conclude that this hair had a higher riboflavin content than that of the control subjects.

Before concluding that this increase is related to the pellagrous condition, however, it would be necessary to eliminate several other causes and clarify certain points. Does the diet of the Yugoslav and the French subject provide the same vitamin content? Moreover, investigation showed that B-complex vitamins had been prescribed for many of these pellagrous subjects. In any event, we found no evidence of riboflavin deficiency in pellagrous hair.

PYRIDOXINE

We used the microbiologic method of determination by means of opacimetric measurement of the growth of Saccharomyces carlsbergensis, according to the technic of Atkin, Schultz, Williams and Frey.[3]

Content of Hair from Normal Subjects. Twenty-seven determinations were carried out on subjects in all age groups, from eight months to eighty-seven years. The results, shown in Table IV cover a rather wide range, with the concentrations varying from 0.07 to 0.46 μg./gm. of hair; the intermediate concentration was, on the average, about 0.20 μg. It is difficult to establish a relationship with the age of the subject, or the color or thickness of his hair.

TABLE III
Riboflavin Content of Hair from Pellagrous Subjects After Five Months

Age (yr.)	Sex	Onset of Disease (yr.)	Color	Texture	Vitamin Content (μg./gm. of hair) (fat removed)
6	F	...	Golden blond	Fine	0.93
7	F	...	Blond	Fine	0.40
9	M	...	Medium brown	Medium	0.42
13	M	...	Medium brown	Fine	0.56
18	M	...	Dark brown	Thick	0.64
20	M	3	Medium brown	Medium	1.90
24	M	3	...	...	2.56
30	F	5	Dark brown	Fine	1.35
30	F	1	Black	Thick	2.15
30	F	5	...	...	1.79
33	F	7	Jet black	Thick	3.32
39	F	10	Dark brown	Thick	3.80
40	F	3	Black	Thick	1.41
43	F	10	Dark brown	Fine	2.89
45	F	5	Dark brown	Medium	2.81
50	F	3	Dark brown	Very fine	1.03
60	F	10	Dark brown	Rather fine	1.12
65	F	3 wk.	Medium brown	Thick	0.17
67	F	5	White	Thick	0.46
Average					1.5

Content after seven months of storage. After seven months of storage at room temperature, in the dark, some samples were again subjected to quantitative determinations, which yielded the following results: before storage, 0.46, 0.12, 0.19, 0.13 and 0.11 μg./gm. of hair; after storage, 0.08, 0.03, 0.06, 0.02, 0.02 and 0.02 μg./gm. of hair.

The average figure after cutting was 0.18 μg./gm. of hair, which dropped to less than 0.04 after seven months. The loss is therefore considerable, i.e., about 80 per cent.

Content of Hair from Pellagrous Subjects. The determinations were carried out on thirteen samples seven months after cutting. The results, given in Table v, range from 0.06 to 0.46 μg./gm. of hair, the average being 0.15 μg.

The average obtained is almost the same as that observed in hair from normal subjects. Actually, if an 80 per cent loss is assumed after seven months, as indicated, the initial content of hair from pellagrous subjects would be higher than that determined in normal subjects. However, the same reservations must be made as in the case of riboflavin. We have no comparative data regarding French and Yugoslav control subjects, and the administration of vitamins could have been a factor in some cases. Nevertheless, the hair of pellagrous subjects does not seem to be deficient in pyridoxine.

PANTOTHENIC ACID

Pantothenic acid is of particular interest since, in certain pathologic conditions, it exerts a favorable effect on hair growth. We used the microbiologic method of L. arabinosus growth developed by Skeggs and Wright,[4] determining the free pantothenic acid content. Petroleum ether was used for the fat removal; extraction was effected after grinding with Fontainebleau sand (about 1,500 mg./100 mg. of hair from which the fat was removed) and washing in the autoclave with distilled water for fifteen minutes at 120°c. After filtration, the pH was adjusted to 6.8 and the suitable dilutions were prepared.

Content of Hair from Normal Subjects. A study by Stangl[5] in 1952 established the

TABLE IV

Pyridoxine Content of Hair of Control Subjects

Age (yr.)	Sex	Color	Texture	Pyridoxine Content (μg./gm. of hair) (fat removed)
8/12	M	Brown	Fine	0.24
9/12	M	Ash blond	Very fine	0.29
1½	F	Light blond	Very fine	0.07
3	M	Light brown	Very fine	0.46
5	M	Auburn	Fine	0.35
15	F	Auburn	Fine	0.32
22	F	Dark brown	Thick	0.42
24	M	Dark brown	Fine	0.22
26	M	Dark brown	Thick	0.41
30	F	Jet black	Medium	0.13
30	F	Dark brown	...	0.33
32	F	Black	Medium	0.12
35	F	Black	...	0.29
51	F	Black	Medium	0.32
55	F	Black	Very fine	0.14
57	F	Dark brown	Fine	0.25
68	M	White	Fine	0.19
71	F	Gray	Fine	0.19
75	F	Silver-white	Fine	0.22
77	F	White	Very fine	0.13
79	F	Gray	Fine	0.42
79	F	Blond	Very fine	0.12
80	F	White	Fine	0.11
83	F	Gray	Medium	0.36
84	F	Black	...	0.09
85	F	White	...	0.17
87	F	Gray	Thick	0.21
Average				0.22

presence of free pantothenic acid in concentrations of 0.05 to 2 μg./gm. of hair. Our own study covers thirty-eight control subjects, the results obtained being listed in Table VI.

The range of concentrations we found is somewhat less broad than that reported by Stangl. They extend from 0.43 to 2.99 μg. of free pantothenic acid/gram of hair, the majority of the concentrations being between 0.60 and 2, with an average of 1.3 μg./gm.

The subjects were between sixteen months and eighty-nine years of age, and their hair color varied. We did not find age or color or thickness of the hair to be of any particular significance. In passing, we might note the relatively high content of free pantothenic acid in hair as compared with that found in the blood. In fact, Gounelle and Richet[6] found an average concentration of 4.6 μg./100 cc. of serum, which corresponds to a content approximately twenty-five times lower than that of the hair.

Content after three months of storage. On the whole, three months of storage in the dark, at room temperature, is followed by a loss in content. We tested nine samples, the average figure obtained being 1.1 μg./gm. of hair at the beginning and 0.82 μg./gm. after three months, i.e., a decrease of about one fourth. However, this drop is not constant; thus, the first two samples maintained practically unchanged concentrations, and for sample No. 8, the loss was only 10 per cent.

The following results were obtained: before storage, 1.07, 1.13, 2.42, 1.56, 0.90, 1.09, 0.83, 0.73 and 0.72 μg./gm. of hair; after storage, 1.01, 1.24, 1.78, 0.79, 0.50, 0.71, 0.55, 0.67 and 0.49 μg./gm. of hair.

TABLE V

Pyridoxine Content of Hair from Pellagrous Subjects (Seven Months After Cutting)

Age (yr.)	Sex	Color	Texture	Pyridoxine Content (μg./gm. of hair) (fat removed)
12	F	Golden blond	Fine	0.06
24	F	Jet black	Thick	0.08
28	F	Black	Medium	0.08
30	F	Brown	Very fine	0.19
30	F	Dark brown	Fine	0.20
31	F	Dark brown	Fine	0.21
34	F	Dark brown	Fine	0.20
50	F	Auburn	Medium	0.08
50	F	Black and white	Medium	0.07
60	F	Dark brown	Fine	0.18
60	F	Light brown	Medium	0.46
60	F	Auburn	Medium	0.07
65	F	Black	Thick	0.09
Average				0.15

Content of Hair from Pellagrous Subjects. The hair of pellagrous Yugoslav subjects was obtained in September 1958, but the determinations were not made until three months later.

The concentrations obtained, shown in Table VII, were between 0.5 and 2.88 μg. of free pantothenic acid/gram of hair, with an average of 1.3 μg. Here, too, we did not find the age of the subject or the color or thickness of his hair to be of any particular significance.

The value of 1.3 μg. shows that there is no decrease in the free pantothenic acid content of pellagrous hair. Taking into account the storage period which, as we have seen, generally results in a reduction in concentration, it is conceivable that the initial concentration

TABLE VI
Free Pantothenic Acid in Hair of Normal Subjects

Age (yr.).	Sex	Color	Texture	Content (μg./gm. of hair)
1 1/3	F	Blond	...	0.98
1 1/2	F	Light brown	...	0.97
11	F	Dark brown	Fine	1.54
12	F	Blond	Fine	0.74
15	F	Blond	Fine	1.18
19	M	Light brown	Fine	2.01
19	F	Black	...	1.94
19	M	Dark brown	Thick	2.21
19	M	Dark brown	Thick	2.23
19	F	Dark brown	Thick	2.45
19	F	Jet black	Thick	2.99
22	F	Dark brown	Thick	2.13
22	M	Ash brown	Medium	1.74
22	F	Blond	Fine	0.85
24	F	Black	Thick	1.13
25	F	Dark brown	Medium	1.04
25	F	Dark brown	Thick	0.63
25	F	Black	...	0.74
26	F	Ash blond	Fine	1
26	M	Dark brown	Thick	2
26	F	Medium brown	Fine	0.51
28	M	Black	Medium	0.81
28	F	Dark brown	...	1.12
29	M	White and much black	Thick	1.07
29	F	Jet black	Thick	0.83
30	F	Medium brown	Fine	0.84
30	F	Black	Medium	1.34
31	F	Ash blond	Fine	0.43
70	F	Gray	Thick	0.73
76	F	White, some black	Thick	1.09
82	M	White, some black	Fine	1.56
82	F	White, some black	Medium	0.99
83	F	White, some black	Thick	0.81
85	F	Very white	Fine	1.14
86	F	Dark brown	Fine	0.83
88	F	White, some black	Medium	2.42
89	F	Ash gray	Medium	0.72
Average				1.28

might have been higher, although still within normal limits.

CONCLUSIONS

We determined the content of four B-complex vitamins in hair from pellagrous subjects, first determining the content of these same vitamins in the hair of normal subjects. Since the determinations in the case of the pellagrous subjects could not be made until several months after the samples were taken, a comparative determination was carried out after a similar period of storage of the control hair.

The niacin content of hair from pellagrous subjects was found to be definitely lower in comparison with that of the control subjects.

TABLE VII
Free Pantothenic Acid in Hair of Pellagrous Subjects

Age (yr.)	Color	Texture	Content (μg./gm. of hair)
40	Black	Fine	1.14
27	Dark brown	Fine	1.36
25	Medium brown with glints of red	Fine	2.17
37	Brown	Fine	0.91
80	Very light blond	Very fine	2.88
80	Dark brown and white	Very fine	0.81
30	Dark brown	Fine	2.71
5	Golden blond	Fine	0.61
65	Black and white	Medium	0.77
7	Brown	Fine	0.84
9	Auburn	Medium	1.11
40	Dark brown	Fine	2.17
28	Jet black	Thick	0.17 (?)
27	Light brown	Fine	1.30
70	Black and white	Thick	0.56
Average			1.3

Storage for three months does not cause any decrease in niacin content.

As for riboflavin and pyridoxine, storage causes a loss of sixty and eighty per cent of the initial content, respectively, after five months of storage for riboflavin and seven months for pyridoxine. The content of these vitamins in hair from pellagrous Yugoslav subjects reflected an increase rather than a decrease.

After three months of storage, the free pantothenic acid in the hair from control subjects decreased by about one fourth. In hair from pellagrous subjects in comparison, the content was not found lower.

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The Relationship Between Diet and Atherosclerosis in Ceylon

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THERE is a large volume of evidence to indicate that nutritional abnormalities are potent etiologic factors in the production of atherosclerosis. Keys[1] and others have postulated that the amount and nature of the dietary fat may be factors of greater importance than others in the production of arterial disease. In support of this argument it is pointed out that coronary thrombosis rarely occurs in countries where the daily consumption of fat is 50 gm. or less, and that autopsy studies in countries where the per capita consumption of fat is relatively low show that atherosclerosis is far less frequently seen than in the United States. More recently this concept has been strongly contested by several workers.[2,3] As Ceylon has been mentioned as one of the countries where the incidence of artherosclerosis is low,[3] it was thought appropriate to summarise the data regarding the intake of various dietary constituents by the Ceylonese and to attempt to correlate this with what is presently known about the incidence of atherosclerosis in Ceylon. The dietary surveys carried out during the past twenty years[4,5] have in the main dealt with the poorer economic classes who form the bulk of the population. As information regarding the diet of the middle class is relatively meagre the results of a survey carried out at two University Hostels are included to give an idea as to the sources of some of the dietary constituents in the Ceylonese diet.

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EXPERIMENTAL STUDIES AND RESULTS

The object of this study was to determine the adequacy of consumption of the various nutrients by residents at the University Medical Hostels and the main sources of food from which the nutrients were obtained. The subjects were undergraduates living at two hostels for medical students maintained by the University of Ceylon at Colombo. All expenses incurred in administering these hostels are met from fees paid by the students (85 rupees per student per month‡). The menu for each day is drawn up by one or more students and the food is bought either daily or weekly from local stores. The purchasing and the cooking of the food are under the supervision of a graduate warden assisted by a student manager, the amount for each item purchased being limited to what is judged, through several years' experience, to be sufficient for the number to be fed at each meal. Wastage is thus reduced to a minimum. A daily record was kept of foods purchased during a period of four weeks; the weights of the items were totaled at the end of this period and the amounts of various dietary constituents consumed per head per day calculated by using standard tables,[6,7] due allowance being made for the waste factor for each item. Allowance has also been made for absence from meals and for the presence of visitors, if any.

There were sixty-nine subjects at the men's hostel and thirty-one at the women's hostel. Their average age, height and weight were, respectively, 23 years, 168 cm., and 55.5 kg. (for

‡ Five Ceylon rupees = one American dollar (approximately).

men) and 26.6 years, 157 cm., and 45.7 kg. (for women). The hostel employees who lived on the premises and ate the same food have been included in the study. Five of them were over thirty years of age. These average heights and weights are greater than the values obtained for the adult population during a survey of physical fitness in the country.[8]

Table 1 indicates the main sources of nutrients taken by the students. As there is an inevitable wastage of food at such institutions, which is not usual in the average household and which, in this instance, was estimated to vary from 3 to 8 per cent of the food prepared per day, a fraction of 5 per cent has been subtracted from the totals in the table. Most of the coconut oil purchased was used in frying and a major portion of the oil was not actually consumed. Half the calories and fat derived from this oil has therefore been subtracted. The intakes of the different nutrients corrected for these two factors are given in the last row of the table.

Most of the milk consumed was in the form of sweetened, evaporated milk taken with tea and with coffee. Women students drank more fresh milk than the men. Animal foods other than milk included eggs, fish (both fresh and dried), beef, mutton, poultry, organ meats, pork, crabs, prawns, and bacon. Both locally produced, par-boiled, lightly milled rice and the weekly ration of highly polished imported rice were cooked; the women ate more of the former variety. In general, rice was taken at the noon meal, while bread or some other preparation of wheat or rice flour was taken at breakfast, afternoon tea, and dinner. The dried pulses cooked included lentil, green gram (mung), green peas, and black gram; lentil was the most popular. A variety of leafy vegetables were eaten, the most popular was cabbage. Under "other vegetables" are included beans, gourds, and other fruit vegetables as well as roots and tubers. Some of the vegetables such as carrots, lettuce, onions, and onion leaves were eaten raw, but most of the vegetables, beef, and pulses were cooked in coconut milk which was obtained by mixing grated coconut with water and manually squeezing out the milk; the process being repeated twice. This milk has been estimated to contain more than 80 per cent of the fat and protein in the kernel. No allowance has been made in the tables for the amount of fat and of protein lost in the discarded fibrous residue, a correction which is not usually made in nutritional surveys. In some preparations the grated coconut was consumed as such.

The calorie intake of the men is below that recommended by the Food and Agricultural Organisation (2,580 calories) for men of similar body weight living in the tropics.[9] Similar low intakes (average 2,005 calories) were found during a previous study on students, in which the food consumed by each person was weighed for several weeks.[10] The intake of women students (2,093 calories), on the other hand, was higher than the allowance of 1,818 calories per day recommended by the Food and Agricultural Organisation for sedentary women of 45 kg. body weight.[9]

Proteins provided 13 per cent (for men) and 10 per cent (for women) of the total calories. Of the 76 gm. of protein ingested daily by the men, 45 per cent was from animal sources and 29 per cent from cereals. The corresponding values for the women were 29 per cent and 52 per cent, respectively. The protein intake of both groups is well above the allowance of 0.58 gm. per kg. body weight recommended for adults living on a mixed rice diet.[11]

The total fat intake by the men was 77 gm. per day, 53 per cent being derived from coconut and 34 per cent from animal sources. The women consumed 57 gm. of fat, 51 per cent from coconut and 38 per cent from animal sources. Fat contributed 30 per cent and 24 per cent of the calories at the men's and women's hostels, respectively.

The calcium intake at the men's hostel was adequate, but less than the suggested allowance of 10 mg. per kg. body weight[10] at the other hostel. A considerable portion of the calcium ingested by the men was derived from small fish and from salted and dried fish which could be fried and eaten whole, bones included. Dried fish was less popular with the women who, therefore, depended on milk for most of the calcium. Vegetables and condiments

TABLE I

THE DAILY PER CAPITA INTAKE OF NUTRIENTS AND THE SOURCES FROM WHICH THE NUTRIENTS ARE OBTAINED

Item	Calories	Protein (gm.)	Fat (gm.)	Calcium (mg.)	Iron (mg.)	Vitamin A (I.U.)	Thiamine (mg.)	Vitamin C (mg.)	Cost (rupees)	Weight Purchased (oz.)
A. Men Students										
Milk and milk products	189	3.1	12.5	118	0.06	486	0.90	0.3	0.160	2.07
Other animal foods	284	32.7	15.8	258	4.69	1,921	0.13	...	0.520	6.17
Rice and rice flour	630	11.7	3.1	18	1.58	...	0.11	...	0.074	6.54
Bread and wheat flour	373	11.4	2.0	23	0.61	10	0.10	...	0.084	4.32
Dried pulses	70	4.6	0.6	15	1.18	27	0.12	0.7	0.020	0.65
Leafy vegetables	10	0.8	0.1	51	0.77	1,712	0.02	24.6	0.031	1.84
Other vegetables	101	6.1	0.8	58	4.24	652	0.13	41.6	0.177	9.80
Coconut	320	3.8	31.0	8	1.60	...	0.05	...	0.068	0.29*
Coconut oil	221	...	24.5	...	...	...	...	...	0.042	0.84
Condiments	51	2.7	1.8	99	1.90	493	0.05	1.7	0.044	1.31
Sesame seeds and oil	11	0.3	1.0	22	0.17	...	0.02	...	0.003	0.07
Sugar and preserves	259	1.8	1.0	...	...	...	...	0.2	0.110	2.45
Fruits	77	1.0	0.4	11	0.45	248	0.03	18.4	0.100	6.10
Tea and coffee	...	...	...	...	...	...	...	...	0.044	0.30
Total	2,596	80.0	94.6	681	17.3	5,549	1.66	87.5	1.48	...
Corrected total	2,356	76.0	77.3	648	16.4	5,272	1.49	73.1	...	...
B. Women Students										
Milk and milk products	233	3.2	17.3	112	0.07	668	0.06	0.3	0.178	2.32
Other animal foods	97	12.0	5.1	16	1.60	1,037	0.07	...	0.415	3.22
Rice and rice flour	627	11.5	2.7	19	1.61	...	0.17	...	0.102	6.15
Bread and wheat flour	521	16.2	2.4	33	0.98	...	0.14	...	0.102	5.66
Dried pulses	24	1.6	0.1	4	0.40	7	0.04	0.2	0.008	0.24
Leafy vegetables	7	0.5	0.1	24	0.47	1,095	0.02	11.8	0.016	1.15
Other vegetables	80	2.9	0.3	43	1.50	502	0.10	26.9	0.116	6.55
Coconut	275	3.3	26.6	7	1.33	...	0.04	...	0.044	0.24*
Coconut oil	73	...	8.2	...	...	...	...	...	0.011	0.29
Condiments	17	0.7	0.6	28	0.97	38	...	0.3	0.058	0.74
Sesame seeds and oil	5	...	0.1	...	...	...	...	...	0.005	0.002
Sugar and preserves	211	...	...	2	0.17	...	...	...	0.084	2.12
Fruits	71	1.0	0.4	9	0.47	214	0.04	12.9	0.214	3.75
Tea and coffee	...	...	...	...	...	...	...	...	0.041	0.26
Total	2,241	52.9	63.9	297	9.57	3,561	0.68	52.4	1.39	
Corrected total	2,093	50.3	56.6	282	9.09	3,383	0.65	49.8		

* Number of nuts.

TABLE II
The Daily Intake of Nutrients by Ceylonese at Different Economic Levels

	A	B			C		D	E	
	Nicholls and Nimala-suriya	Cullumbine et al.			Present Investigation		High Income Groups in Colombo	European Residents in Ceylon	
		Group I	Group II	Group III	Men	Women		Men	Women
Calories, total	2,296	2,163	2,469	3,112	2,356	2,093	3,271	2,700	1,910
From protein (%)	10.4	14.2	12.2	11.8	12.9	9.6	10.3	13.1	13.8
From fat (%)	14.3	14.3	18.9	26.5	29.5	24.3	26.1	30.5	35.4
Fat, total (gm.)	36.5	39.8	49.7	91.6	77.3	56.6	95	91	75
Animal (gm.)	1.6	4.4	6.7	19.5	26.9	21.3	36.2	60.5	57.1
Coconut (gm.)	25.0	24.0	25.0	37.5	41.0	29.0	48.6	...	...
Proteins, total (gm.)	59.5	54.0	74.3	85.1	76.0	50.3	84	88	66
Animal (gm.)	24.4	27.2	26.0	25.1	34.0	14.4	42	56.2	54.8
Carbohydrate (gm.)	430	387	430	480	337	360	520	380	242
Calcium (mg.)	503	362	560	683	648	282	762	826	623

NOTE: A, Calculated from results of Nicholls and Nimalasuriya;[4] B, calculated from results of Cullumbine et al.;[5,12] D, calculated from reference 14; and E, calculated from results of Cullumbine.[15] Results in columns A, B, and E are expressed in terms of adult consumption unit. The other columns give the per capita intake.

contribute significantly to the calcium intake among the Ceylonese. Minerals derived from tea, coffee, and crude kitchen salt have not been taken into account in the tables. Kitchen salt has been estimated to contribute from 20 to 40 mg. calcium per day.[12] In the absence of animal foods the intake of calcium would be reduced considerably (150 to 200 mg. per day). Such low levels have been reported by previous investigators[10] who have suggested an adaptation by the population to low calcium intakes. The iron intake at the men's hostel was adequate, while it barely equaled the suggested allowance of 9 mg. per day[12] at the women's hostel.

Most of the vitamin A is ingested as its precursor, carotene. When the values are expressed in terms of the "preformed vitamin" by reducing all carotene values by two-thirds,[13] the daily intakes amount to 3,282 I.U. (for men) and 2,231 I.U. (for women). The latter value is below the suggested allowance of 2,592 I.U.[12] A similar low intake has been reported for the general population.[12]

The diet consumed by the students at these two hostels is comparable to that of the middle class in Ceylon. It is adequate in calories and in proteins. The intakes in calcium, iron, and vitamin A tend to be low when the intake of animal foods is minimum, and when cheap sources of these nutrients, such as small fish, dried fish, and leafy and other vegetables, are not popularly eaten. The total fat intake tends to increase with increasing consumption of milk and butter.

COMMENTS

The results of some of the nutritional surveys carried out in Ceylon during the past twenty years are summarised in Table II. Column A gives values calculated from the data obtained by Nicholls and Nimalasuriya in 1940 during a survey of the nutrition of 409 families living in rural areas in the country. The survey carried out by Cullumbine and others[5,12] in 1948–1949 included both urban and rural areas. The subjects were divided into three groups according to the income per adult consumption unit: namely, those earning less than 20 rupees per month (group I in

column B), those earning between 20 and 50 rupees (group II) and those whose income was above 50 rupees (group III). The results in column B are expressed in terms of adult consumption unit per day. The values in column D have been calculated from the published results of a survey of seventy-two families in the city of Colombo with an income of over 500 rupees per month per family, carried out by the Medical Research Institute in conjunction with the Department of Census and Statistics,[14] and would be representative of the richer class in Ceylon. Column E gives values obtained by Cullumbine in a survey of the diets of European residents in Ceylon,[15] also expressed in terms of adult consumption unit.

It will be seen that the calorie intake of the poorer classes, who form the majority of the population and most of whom are manual workers, falls short of the recommended allowance[9] (column A and groups I and II of column B). In contrast, 88 per cent of the families in the high income group (represented by column D) were found to exceed their calorie requirement by about 150 per cent.[14] In all groups, proteins provide 10 to 14 per cent of the calories, whereas the contribution from fat increases from 14 to 30 per cent with increase in income.

There are no vegetarians in Ceylon. The Buddhists who form more than 70 per cent of the population will drink milk. A majority of them eat fish, and many of them consume eggs, beef, and mutton. The Hindus consume more milk and milk products than the Sinhalese Buddhists. Most of them eat fish and mutton. The Muslims avoid only pork, and the animal foods eaten by the Christians are limited only by the family income. As a majority of the population belongs to the lower income groups, their intake of fat would be 35 to 50 gm. per day and about 80 per cent of this would be coconut fat. Due to a short supply of good quality rice, the protein quality would be unsatisfactory unless the diet was well supplemented by foods such as dried pulses and fish.

The Incidence of Atherosclerosis

Although no values for serum cholesterol levels among the population have been published and no systematic study of autopsies have been reported to give an indication as to the incidence of atherosclerosis in Ceylon, some information regarding the occurrence of arterial disease is available.

Fernando and Wijenaike[16] studied the case histories of patients admitted to the General Hospital, Colombo, under their care during the period 1952–1953, and concluded that the occurrence of coronary artery disease was similar to that in temperate climes with respect to frequency, age distribution, and severity of the disease. Of a total of 5,944 patients, 298 (or 5 per cent) were admitted for heart disease. Of these 298, 24.5 per cent suffered from ischaemic heart disease, 71 per cent of whom were over fifty years of age and the ratio of males to females was 2:1. These figures, they point out, are comparable to those reported for Great Britain. Although it is surmised by the authors that, since these patients were admitted to the "nonpaying" wards of the hospital, they would belong to the lower and middle economic classes, it is not known what proportion of the 1.25 per cent of total patients admitted suffering from ischaemic heart disease belonged to the higher income groups.

From a study of 100 patients known to have myocardial infarction Wijesekera[17] concluded that "infarction was less frequently met with in patients who ate well," whereas the condition was apt to develop in those who consumed a minimum of eggs, meat, and fatty foods. The lowest number of infarctions were seen among the Muslims who "are known to consume more animals foods than the Sinhalese or Tamils."

The results of a series of ninety consecutive postmortem examinations carried out by the Department of Pathology of the University of Ceylon during the period 1956–1957 are given in Table III. The material for these autopsies was derived from the "nonpaying" wards of the General Hospital, Colombo. There is considerable difficulty in obtaining permission from the relatives of the diseased for postmortem examination; for this reason the higher income groups would not be represented in this series, a majority of the autopsies at

TABLE III

The Incidence of Atheroma as Shown by a Series of Ninety Consecutive Autopsies Performed at the General Hospital, Colombo

Age (yr.)	Males		Females	
	Aorta	Coronary Arteries	Aorta	Coronary Arteries
<21	2/4	1/4	0/1	0/1
21–25	2/3	1/2	1/1	1/1
26–30	0/1	0/1	2/3	0/3
31–35	2/4	1/4	3/3	1/3
36–40	6/7	4/7	2/2	1/2
41–45	5/5	4/5	2/2	1/2
46–50	6/6	2/3	2/2	1/2
51–55	2/2	2/2	1/1	1/1
>55	30/31	21/26	12/12	10/11
Total	55/63	36/54	25/27	16/26
Circle of Willis affected	6 of 18 examined		3 of 4 examined	

NOTE: No attempt has been made to grade the degree of the atheromatous change. The coronary arteries were examined in eighty and the Circle of Willis only in twenty-two of these cases.

the Ceylon Hospitals being performed on subjects belonging to the poorer classes. Of the ninety patients studied, the coronary arteries had been examined in only eighty and the circle of Willis in only twenty-two. In all cases the aorta had been examined for evidence of atheromatous change. The results are arranged according to age and sex; in each group the number of patients with atheroma is indicated in the numerator and the total number examined in the denominator. It will be seen that among the males fifty-five of sixty-three aortas had some degree of atheroma, and thirty-six of fifty-four coronary arteries and six of eighteen cerebral vessels were similarly diseased. Among the females, twenty-five of twenty-seven aortas, sixteen of twenty-six coronary vessels and three of four cerebral vessels were found to be affected. In all these cases the disease varied from a few specks of fatty deposits in the intima to large calcified and ulcerated patches. In only two of our ninety cases was atherosclerosis the cause of death.

This study of autopsies is admittedly inadequate. The number is small and no attempt has been made to estimate with any degree of accuracy the extent of the damage to the arterial wall and to record the income and particulars regarding the dietary habits of the deceased. For a comparison to be made with the incidence of atherosclerosis in other countries it will be necessary to classify the cases studied according to the degree of the atheromatous change.[18] A further objection is that these autopsies were performed on people who have died of some illness or other and could not therefore represent the condition of the arteries in the healthy population. Examination of the arteries of those who die suddenly due to accident or violence would therefore provide valuable evidence. A more detailed study is now under way. However, these figures taken in conjunction with reports of autopsies from other Asian countries[2] give the impression that atherosclerosis in countries like Ceylon is not as rare as it is generally supposed to be. It would be appropriate, therefore, to inquire into the possible "atherogens" in the Ceylonese diet.

Dietary Fat

That the changes in the arteries is not due to a high fat intake is indicated by Table II. The dietary fat is, however, made up mainly of coconut fat which is a highly saturated fat; and saturated fat, whether of animal or of vegetable origin, has been shown to increase serum lipids.[19] Further, coconut fat has a high proportion of lauric acid (about 45 per cent), and glyceryl trilaurate has been shown to produce a considerable rise in serum cholesterol in rabbits.[20] When this ester constitutes 8 per cent of the diet its hypercholesteremic effect cannot be completely abolished by adding 4 per cent corn oil or linoleic acid. This result is interesting in view of the fact that the average Ceylonese diet does not contain a high proportion of polyunsaturated fatty acids such as linoleic acid. A small amount of sesame oil is used in the preparation of some of the foods eaten by the Tamils (who form about 15 per cent of the population) but the amount consumed is strictly limited owing to the high cost of this oil (see also Table I). It will be

seen from Table II that a certain amount of it (varying from 10 to 18 gm. daily among the low income groups) is derived from vegetable sources other than coconut and the nature of the fatty acids in these foods is not known.

Dietary Protein

In a recent review Olson[21] points out that serum lipid levels are low among those suffering from protein malnutrition, and that atherosclerosis is less commonly found at autopsy among patients with cirrhosis than among those without. He concludes that the intake of animal protein plays a significant part in determining the rate of atherogenesis in man. In a deficiency of lipotropic factors in the diet, liver fat increases while serum lipid levels fall; therefore, atherosclerosis is less likely to develop among those on a minimal animal protein intake.[21] On the other hand Fernando[22] has reported a prevalence of cirrhosis following fatty infiltration among patients admitted to the General Hospital, Colombo, and has ascribed this incidence to a deficiency of lipotropic substances in the diet. These patients are part of the same population in whom a high incidence of ischaemic heart disease was found.[16] In the autopsy cases reported in Table III there were eight patients in whom the liver showed fibrosis; of these six had marked atheromatous changes in the arteries. What proportion of the patients had fatty changes in the liver not apparent to the naked eye are not known. There is nothing in our findings, however, to support Olson's postulate that a high protein intake is an important etiologic factor of atherosclerosis.

Dietary Carbohydrate

Reports from a few investigators indicate that a high carbohydrate intake might contribute to raised serum lipid levels and atherogenesis. Prominent among these is Gofman[23] who has shown that increasing the quantity of dietary carbohydrate from 220 to 385 gm. per day, with a total calorie intake of 2,350 per day, produces a marked rise in the level of $S_f°$ 20–400 serum lipoproteins. The poorer classes in Ceylon consume between 380 and 480 gm. carbohydrate daily (Table II) and could be expected to have high levels of these serum lipoproteins. The richer classes not only take an excess of carbohydrate but also have a high fat intake, a considerable portion of which is made up of saturated fats from coconut and from animal sources. Among them, therefore, there should be a high level of low-density serum lipoproteins as well as lipoproteins of the class $S_f°$ 20–400,[23] and they would be more liable to suffer from thrombosis of sclerosed arteries. No large-scale investigation of serum lipids have been carried out in Ceylon. A study at the Lady Ridgeway Children's Hospital, Colombo, showed that the blood cholesterol level among children suffering from "fatty liver disease" was 161.6 ± 8.83 mg. per 100 ml. (range 110 to 341 mg. per 100 ml.) while that among the rest of the children admitted to the Hospital was 149 mg. per 100 ml. (range 103 to 203 mg. per 100 ml.).[24] Phospholipid values in children vary from 4.3 to 12.2 with an average of 8.8 mg. phospholipid phosphorus per 100 ml. plasma.[25] For the adult population values between 180 to 240 mg. total cholesterol per 100 ml. serum are taken as normal. Reduced levels of serum cholesterol and of S_f 0–12 beta-lipoproteins with normal or increased concentrations of the S_f 12–100 beta-lipoproteins have been found amoung Central Americans and Costa Ricans[26] who appear to subsist on diets similar to those taken by the poorer classes in Ceylon.

SUMMARY

If, therefore, the incidence of atherosclerosis in Ceylon is significant, as is suggested by the few autopsy cases cited, and by the findings of Fernando and Wijenaike,[16] the dietary factors responsible might be either a high intake of carbohydrates or an insufficient intake of unsaturated fatty acids such as linoleic acid which are believed to counteract the effect of relatively high intake of fatty acids such as lauric acid[21,27]; or a combination of both factors might be responsible. A high intake of fat or of protein is not likely to be an etiologic factor. On the other hand the question has also been raised as to whether a deficiency of lipotropic factors may not lead to a fatty

change in the intimal layer of the arteries and thus bring about an atheromatous change.[16]

ACKNOWLEDGEMENT

We wish to thank Dr. N. D. Gunasekera for permission to include his results in Table III. We also thank Prof. G. H. Cooray for helpful criticism of the paper, the Resident Wardens of the two University Hostels for their kind cooperation and the medical students Agatha Phillips and Malini Weerasekera, V. Venkateswaran and A. Mageson for their valuable assistance in the compilation of the data.

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Studies of Serum Desmosterol Levels in Hypercholesteremic Subjects Treated with Triparanol (MER-29)

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THE new approach to the treatment of hypercholesteremic states, i.e., inhibition of cholesterol biosynthesis, has certain real or potential disadvantages. Thus, when the inhibitor acts early in the cholesterol metabolic pathway (as with benzmalecene), there is an accumulation of triglycerides probably due to a diversion of available acetate from cholesterol synthesis to triglyceride synthesis.[1] When the inhibitor acts late in the pathway, i.e., after cyclization of squalene to form the steroid nucleus, an accumulation of a steroid precursor of cholesterol may result. Such a "late" inhibitor is triparanol (MER-29), and indeed, the cholesterol precursor, desmosterol (24-dehydrocholesterol) has been demonstrated in the liver and serum of rats[2] and in the serum of patients[3–5] taking this compound.

We, as others, have been concerned about the appearance and accumulation of desmosterol, which structurally so closely resembles cholesterol, because of the potential danger of atherogenesis from this precursor sterol. Therefore, we have followed the serum level of desmosterol in a prolonged investigation of the effects of triparanol on lipids and lipoproteins. This report will be concerned only with the problem of desmosterol accumulation.

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METHODS

Most of the available chemical methods for the determination of cholesterol determine other sterols as well. If the usual colorimetric analysis for "cholesterol" (which measures both desmosterol and cholesterol) is utilized during triparanol therapy, the specific progress of the serum *cholesterol* concentration cannot be followed. It has been shown that the Zak method,[6] which uses ferric chloride for cholesterol color development measures both total cholesterol and total desmosterol, and that the Abell method,[7] which uses a modified Liebermann-Burchard reagent for color development measures cholesterol plus 52 per cent of the desmosterol.[4] We have found that in our laboratory the Zak method measures total cholesterol and 80 per cent of desmosterol, whereas the Abell procedure measures total cholesterol plus 56 per cent of the desmosterol. These values have been obtained using both desmosterol‡ and cholesterol standard solutions, individually and in combination. Thus, by the following algebraic formula§ it is possible to determine the desmosterol content of serum:

$$\begin{aligned} Z &= C + 0.80D \\ -(A &= C + 0.56D) \\ \hline Z - A &= 0.24D \end{aligned}$$

where

Z = mg. % sterol as determined by the Zak method,
A = mg. % sterol as determined by the Abell method,
C = cholesterol, and D = desmosterol.

We have used these technics in studying the serum of ten patients with disorders of lipid metab-

‡ The desmosterol was kindly supplied by Dr. T. R. Blohm of Wm. S. Merrell Co.

§ The formula represents a modification of relationships proposed by Frantz et al.[4]

TABLE I

Effect of Triparanol (MER-29) Administration on Serum Cholesterol and Total Sterol Concentrations

Patient	Diagnosis	Control Cholesterol Average* (mg./100 ml.)	Triparanol Therapy							
			Cholesterol				Total Sterol			
			Average†		Minimal Value		Average		Minimal Value‡	
			mg./100 ml.	% Fall	mg./100 ml.	% Fall	mg./100 ml.	% Fall	mg./100 ml.	% Fall
M. K.	Xanthoma tendinosum	547	415	24	386	30	469	14	465	15
B. T.	Familial hypercholesteremia	600	308	49	244	59	413	31	428	29
S. T.	Familial hypercholesteremia	415	216	48	188	55	318	23	326	22
G. B.	Familial hypercholesteremia	484	267	45	171	65	392	19	427	12
I. F.	Familial hypercholesteremia	399	300	25	230	42	370	7	358	10
M. H.	Xanthoma tuberosum	614	293	52	230	63	338	45	284	54
R. J.	Nephrotic syndrome	654	393	40	304	54	630	4	624	5
E. F.	Coronary artery disease	444	336	24	261	41	437	2	395	11
E. W.	Xanthoma tuberosum	447	318	29	285	36	412	8	393	12
S. R.	Idiopathic hypercholesteremia	354	244	31	202	43	287	19	272	23
Average		496	309	37	250	49	407	17	397	19

* Average of three values taken at weekly intervals.

† Average of seven values taken over three month period, three months after MER-29 therapy started except for E. W. whose average represents a two-month period.

‡ Total sterol (cholesterol plus desmosterol) on same serum which demonstrated maximum per cent fall in cholesterol.

olism, including xanthoma tendinosum and tuberosum (three), familial hypercholesteremia (four), idiopathic hypercholesteremia (two) and nephrotic syndrome (one). The subjects received triparanol,* 1,000 or 1,500 mg. daily in divided doses. These subjects were studied for periods of five to thirteen months and are still under observation.

RESULTS

All subjects demonstrated significant falls in serum cholesterol levels averaging 37 per cent ($p = <0.001$) (Table I). When desmosterol plus cholesterol (hereafter referred to as total sterol) was measured on these same serum samples the apparent fall in "cholesterol" was less (17 per cent) but still significant ($p = <0.01$). The *maximum* fall in true cholesterol levels averaged 49 per cent, and in total sterol concentrations, 19 per cent.

Desmosterol was not demonstrable in the serum obtained before treatment. During the administration of triparanol, the maximum increase of desmosterol averaged 167 mg. per 100 ml. and ranged from 84 mg. per 100 ml. to 320 mg. per 100 ml. This represented an average of 37 per cent of the total sterol.

In seven of the ten subjects, desmosterol was demonstrable in the serum within two weeks. In the others, desmosterol was not found in the serum until after three, four and eleven weeks of triparanol administration. The time of appearance of desmosterol was not related to dose.

In two subjects there was no desmosterol

* Triparanol was generously supplied by Dr. J. S. Scanlan and Dr. R. H. McMaster of the Wm. S. Merrell Co.

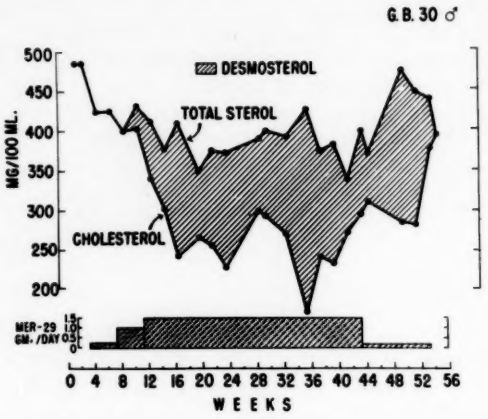

FIG. 1. Appearance of desmosterol in serum of patient with familial hypercholesterolemia during triparanol therapy.

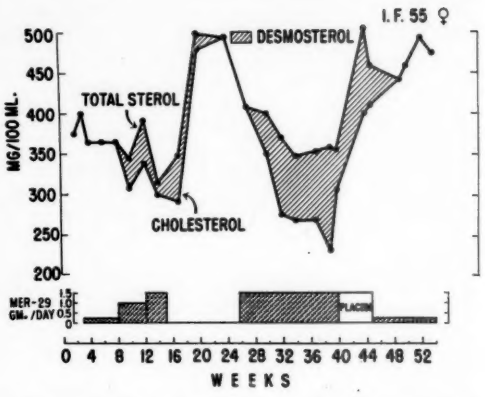

FIG. 2. Effect of interdiction of triparanol therapy on serum desmosterol levels of patient with familial hypercholesterolemia.

in the serum during initial four and eight week periods of 250 mg. triparanol daily, but desmosterol promptly appeared when they were switched to the larger doses (Fig. 1). Aside from these, there appeared to be no correlation between the presence of desmosterol and triparanol dosage. Nor was there a relationship between the duration of the study and desmosterol level, i.e., once a level was established there appeared to be fluctuations around that level rather than progressive accumulation of the sterol.

Following cessation of triparanol therapy (by switching to placebo) in three of the subjects, desmosterol continued to be demonstrable in the serum for five to six weeks (Fig. 2).

COMMENTS

It would appear from these studies that progressive accumulation of desmosterol in the serum of patients on triparanol therapy does not represent an inherent potential danger to the continuation of this mode of therapy. Although Frantz et al.[4] described one patient in whom the total sterol rose during therapy, in our series the rise of serum desmosterol was always associated with a fall in total sterol. If one postulates that desmosterol is as atherogenic as cholesterol, one still would not expect *increased* atherosclerosis as the result of triparanol therapy because the total sterol (desmosterol plus cholesterol) levels never exceeded the initial levels of cholesterol. It is not yet known whether the presence of desmosterol in serum in amounts reported herein can produce harmful effects. Long-term biologic effects of desmosterol are not known, but it would appear that desmosterol is converted to and loses its identity in bile acids in a manner indistinguishable from that of cholesterol.[8]

Of interest is the fact that the concentration of serum desmosterol did not progressively increase. Once desmosterol was demonstrated in the serum, the concentration fluctuated from week to week. It might be expected that a compound which completely inhibits the last step of cholesterol biosynthesis would produce a progressive accumulation of the immediate precursor. Since our data indicate that this does not occur, they suggest that desmosterol itself might act as an effective inhibitor in the chemical negative feed-back system regulating cholesterol biosynthesis.[9] The fact that the serum cholesterol concentration does not fall to zero indicates that inhibition of biosynthesis is incomplete or that an alternative pathway for cholesterol biosynthesis is present and, indeed, one has recently been suggested by Steinberg and Avigan.[10]

It is clearly recognized that the preceding comments must be tempered by the fact that

serum desmosterol is not determined directly by the method presented. The desmosterol values represent the difference between two methods, each controlled with appropriate crystalline desmosterol and cholesterol standards.

SUMMARY

The concentration of desmosterol, the immediate precursor of cholesterol in the synthetic chain, was followed in the serum of ten patients with disorders of lipid metabolism and hypercholesteremia given triparanol (MER-29) for a prolonged period.

All subjects demonstrated falls in total sterol (desmosterol plus cholesterol) levels which averaged 17 per cent. This was significant, but less than the average fall in serum cholesterol of 37 per cent. During triparanol administration, the maximum increase of desmosterol averaged 167 mg. per 100 ml., which represented 37 per cent of the total sterol. Progressive increases in serum desmosterol levels as triparanol therapy continued did not occur. It is not yet known whether the presence of desmosterol in serum in these amounts can produce harmful effects.

ACKNOWLEDGMENT

We are grateful to Dr. Louis Leiter, Chief, Medical Division, and to Dr. J. Philip Savitsky, Research Associate, Montefiore Hospital, New York, for helpful advice and criticism.

ADDENDUM

After this paper was submitted for publication, similar findings were reported in nine normo- or mildly hypercholesteremic subjects.[11]

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Reviews of Recent Books

The Arterial Wall, edited by Albert I. Lansing, Williams & Wilkins Co., Baltimore, 1959, pp. 259, $7.50.

The arterial wall is currently in both public and scientific lime-lights; a book about it deserves and will receive attention. Dr. Lansing has edited a compendium of ten chapters contributed by thirteen distinguished American scientists. Anatomy, biochemistry, physiology, enzymology and metabolism of arterial tissue of the normal vessel are considered, although references are made to changes with age and arteriosclerosis. Even when not stated, it is apparent that the arterial wall in atherosclerosis is not far from the minds of all.

Any book to which a number of investigators contribute inevitably suffers unevenness, overlaps and omissions. But here all appear *in extremis* nearly to the point of caricature. The opening chapter on *vasa vasorum* by Charles Woerner is in essence a report of his studies of some forty human arteries and thirty-five others from dogs or rabbits using clearing and injection methods; he gives brief citations from pertinent literature. But Dr. Wilfried F. H. M. Mommaerts' chapter on arterial muscle is a masterly lucid summary-in-depth of muscle physiology, structure and biochemistry, including contemporary knowledge of proteins of muscular tissue and dynamic aspects of contractile activity of both striated and smooth varieties. This chapter presents not only the fundamentals but also the recent advances in this complex area in a thorough and succinct manner that will certainly command readers' admiring respect. The section dealing with the structural aspects would have been enhanced by light and electron micrographs, but this minor omission cannot obscure the excellence of his presentation, which is one of the best that this reviewer has ever encountered. It is divided into several sections, each with its own separate and extensive bibliography providing many useful references.

On the same high plane is Dr. Zweifach's contribution on vascular endothelium which emphasizes nicely the many areas in which knowledge is lacking, particularly histochemistry and histoenzymology of endothelium in relation to age and species. These deficiencies constitute a handicap in any attempts to assess changes in endothelium with aging or in atherosclerosis. Dr. Zweifach's chapter is highlighted by many excellent key references of the older literature (of fifty years ago and more) as well as recent.

Although the contributions by Woerner, Zweifach, and Mommaerts can be followed by the student who does not work in these specialized areas, that of Batchelor and Levene on collagen and ground substance cannot be grasped by those unfamiliar with the background material (although they do cite key reviews of it). These contributors have addressed themselves to the problem of fiber growth with emphasis on the perifibril zone. It is regretted that these authors were not allowed space to go into background as did some of the others.

The problem of elastic tissue and the enigma of elastase is ably reviewed by the editor, Dr. Lansing, who has made many major contributions to this field. The origin of the enzyme, elastase, is reviewed and efforts to localize it in some specific portion of the pancreas described. Unfortunately, in some of the experiments it has not been appreciated that although cobaltous chloride (at least in guinea pigs) produces damage to alpha cells, it is even more toxic to acinar tissue and conclusions suffer accordingly. In his review of mucopolysaccharides of arteries, Dr. John E. Kirk ably presents the chemistry and histochemistry of the subject. His own work on chromatography of polysaccharides of arteries from man is illustrated with color plates. He points out discrepancies between histochemical and biochemical studies, for example, the increase in mucopolysaccharides in arteriosclerosis indicated by histochemistry has apparently not been confirmed by biochemical estimations. A possible explanation may be that some histochemists have confused mucopolysaccharides with the pigment, ceroid, which has similar staining characteristics and does increase in arteriosclerosis. Barrows and Chow review the rather scanty knowledge concerning enzymes in arterial tissue. They present a limited "birds-eye view" of the available literature, suggesting approaches for further study. Reading their chapter, one is forced to conclude that enzymatic studies of arterial tissues have not yet been particularly rewarding.

Lipid metabolism of connective tissue as related to vessels in aging is reviewed by Drs. Boucek and Noble who apparently believe that initial changes in atherosclerosis consist of deposition of connective tissue and alteration of ground substance before any abnormal lipids appear. This view is not the consensus of all students of the subject, but based on this premise Boucek and Noble have directed their attention to the accumulation of sterols and lipids in fibroblasts growing in implanted sponges in animals and various races of man (White, Negro, Indian and Eskimo). With this

artificial model they have made interesting and stimulating observations which are not, however, easy to interpret in relation to either normal or pathologic arteries. Dr. Lehninger's final chapter on metabolism of the arterial wall to a certain extent reviews aspects of the previous contributions. Dr. Abraham Dury has provided a summary of some five pages neatly pointing out the highlights of the various chapters.

The index provides frequent cross references, the format is pleasing and the print exceptionally easy to read and free of more than but a few typographical errors.

This book provides rather specialized views on certain aspects of normal arteries. Some chapters are suitable only for the advanced student while others are capable of assimilation by any intelligent reader. Documentation is good and the bibliographies will serve well for references to further reading. In the introduction, the editor formulates a number of questions that he believes are suggested by the contents that follow. Should this book stimulate intensified search for answers its purpose will have been served in an exceptionally worthwhile way. This monograph is recommended reading for all investigators in the field.

W. Stanley Hartroft

BOOKS RECEIVED FOR REVIEW

Books received for review by *The American Journal of Clinical Nutrition* are acknowledged in this column. As far as practicable, those of special interest are selected, as space permits, for extensive review.

The Premature Baby, 5th Ed., by V. Mary Crosse. Little, Brown & Co., Boston, 1961, pp. 266, $7.00.

Selected Films and Filmstrips on Food and Nutrition, Food & Nutrition Council of Greater New York, 1961, pp. 72, $1.25.

1960–1961 Year Book of Endocrinology, edited by Gilbert S. Gordan, Year Book Medical Publishers, Chicago, 1961, pp. 408, $8.00.

Progress in Medical Genetics, Vol. 1, by Arthur G. Steinberg. Grune & Stratton, Inc., New York, 1961, pp. 341, $9.75.

Ciba Foundation Study Group No. 7, Virus Meningo-Encephalitis, edited by G. E. W. Wolstenholme and Margaret P. Cameron. Little, Brown & Co., Boston, 1961, pp. 120.

Ciba Foundation Symposium. Quinones in Electron Transport, edited by G. E. W. Wolstenholme and Cecelia M. O'Connor. Little Brown & Co., Boston, 1961, pp. 453, $11.00.

Abstracts of Current Literature

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VITAMIN C DEFICIENCY

A dietary deficiency of vitamin C will cause scurvy only in man, other primates and the guinea pig; other mammals can synthesize their needs. The basis for its requirements in man is uncertain and wide differences are seen in the recommended allowances in various standards. There is no storage of the vitamin, a constant dietary source being necessary.

Infantile Scurvy. M. K. Strelling. *Brit. M. J.*, 1: 701, 1960.

Since the war there have been a number of reports of isolated cases of scurvy in young children; these have usually been quite clearly due to a grossly deficient diet, often in mentally defective children or in children with perverse eating habits. The case is reported here of a fifteen month old boy living in a well run home for deprived children in who a large sub-periosteal hemorrhage of the right lower femur developed, with swollen bleeding gums. Given large doses of ascorbic acid, he recovered completely.

The interesting feature of this story is that the child had received 50 mg. daily of ascorbic acid until he was nine months old and for the following six months a teaspoonful of rose-hip syrup supplying 7 mg. of ascorbic acid, along with a reasonably good mixed diet. Although the level of vitamin C necessary to prevent scurvy in children is not known, it must be not much above 7 mg. and it is suggested that the rest of the diet must have been almost devoid of vitamin C. The case "emphasises how a diet which is relatively fixed, as in an institution, and relies on every day methods of preparing vegetables may be grossly deficient in vitamin C." F. E. HYTTEN

Ascorbic Acid Deficiency in Addisonian Pernicious Anemia. D. Todd. *Scot. M. J.*, 4: 249, 1959.

There was biochemical evidence of ascorbic acid deficiency in half of a group of fourteen patients with classic Addisonian pernicious anemia. In none, however, was there clinical evidence of scurvy. This deficiency was not limited to patients with pernicious anemia, for it was found in five of thirteen patients with other anemia and six of thirteen nonanemic patients. In no instance were clinical features of scurvy observed. In addition, no correlation was found between the severity of the anemia and blood ascorbic acid level.

Ascorbic acid deficiency did not affect the initial hematologic response to therapy with vitamin B_{12} but appeared to have delayed subsequent regeneration of the red blood cells. The author postulates that, by interfering at the folinic acid level, ascorbic acid deficiency disturbs the normal chain reaction concerned in the synthesis of nucleoprotein. Ascorbic acid may play a subsidiary role in erythropoiesis in patients with pernicious anemia. S. O. WAIFE

As in the case of other vitamins studied, alcoholics appear to have a subclinical deficiency with respect to vitamin C. Perhaps this aspect of the problem would be remedied by "Tom Collins."

The Vitamin C Status of Alcoholics. D. Lester, R. Buccino and D. Bizzocco. *J. Nutrition*, 70: 278, 1960.

Knowledge of the magnitude of vitamin C deficiency in alcoholics may help to establish a basis for a rational program of nutritional therapy. Subjects as received at the hospital were either intoxicated or suffering from the effects of prolonged drinking. Prior to the time of admission, the intake of vitamin C was trivial. Healthy nonalcoholics were used as controls. The test doses of vitamin C were 250, 500 or 1,000 mg., which were ad-

ministered daily. Nonalcoholics, saturated with vitamin C, following the administration of 500 mg. vitamin C, excreted in the urine in four hours -34.9 ± 12.3 per cent of intake. Individual variabilities were considerable. The use of twenty-four hour urine collections gave more consistent results; but it was concluded that as a practical simple test the shorter collection period has advantages. It was concluded that any person who excreted less than 10.3 per cent of intake in the four hour test is deficient in vitamin C.

The administration of at least 500 mg. of vitamin C daily for a week was necessary to raise the alcoholics to saturation. A significant proportion of the nonalcoholics were found to be vitamin C-deficient, but the differences were considerable between this group and the alcoholics quantitatively.

The authors recommend that alcoholics be given 500 mg. of vitamin C daily for a week or more, and thereafter 150 mg. daily. FRANK E. RICE

Vitamin C participates in the formation of colloidal intracellular substances of cartilage, dentures and bone, and in the polysaccharides of skin and fibrous tissue. Investigations of vitamin C deficiency states affecting certain of these tissues are of great clinical significance.

Connective Tissue Studies. II. The Effect of Vitamin C Deficiency on Healed Wounds. A. F. Abt, S. von Schuching and J. H. Roe. *Bull. Johns Hopkins Hosp.*, 150: 67, 1959.

The data presented in this paper indicate that vitamin C is necessary for the maintenance of previously formed scar tissue. Mid-line abdominal incisions were made on a group of guinea pigs. Forty-three days later some of the animals were placed on a scorbutic diet for thirty-three days. The strength of the wound tissue was determined by measuring the pressure necessary to cause wound rupture. In the vitamin C-deficient animals an average of 336 mm. Hg was required to rupture the skin and muscle tissue of the wound. The wound's skin and muscle could not be ruptured in the control, nondeficient animals except for one animal. The skin of this animal's wound was ruptured at 490 mm. Hg, but rupture of the wound's muscle was not achieved.

Scar skin and muscle contain a high concentration of ascorbic acid as compared to nonscar skin and muscle. This was true in wounds ten days old as well as in wounds seventy days old. Greater amounts of C^{14}-ascorbic acid were found in the scar tissue as compared to nonscar tissue after injection of the labeled vitamin. This would indicate that part of the high levels of ascorbic acid in wound tissue is obtained from recently administered ascorbic acid. In the one sample of human skin obtained from old scar tissues, ascorbic acid levels were 64 per cent greater than those of normal skin tissue. M. W. BATES

Oral Changes in Normal and Scorbutic Guinea Pigs Injected with Ascorbic Acid-1-C^{14}. S. H. Yale, H. Jeffay, C. I. Mohammed and E. C. Wach. *J. Dent. Res.*, 38: 396, 1959.

Normal and scorbutic guinea pigs were given intraperitoneal injections of tracer doses of ascorbic acid labeled in carbon No. 1 position with radioactive carbon, and sacrificed five minutes to ninety-six hours after injection. Within five minutes, radioactivity was demonstrated in the periodontal membrane, pulp, alveolar periosteum, supporting and cortical bone, liver, adrenal and submaxillary gland of both the normal and scorbutic guinea pigs. However, no evidence of radioactivity was observed in the enamel or dentin at any time interval. The peridontal membrane appeared to be one of the first tissues to incorporate ascorbic acid. The distribution of ascorbic acid in the pulp was fairly uniform except at the periphery where the odontoblastic layer had a greater ascorbic acid uptake than other of the pulp. Not all radioactivity in the pulp was attributable to ascorbic acid but an appreciable amount of the C^{14} released in the metabolism of the tagged ascorbic acid was present in some unknown chemical form. A particular difference between normal and scorbutic guinea pigs was noted in respect to the incorporation of radioactivity into the mandibular cortex. The higher level of uptake in the mandibular cortex of the normal guinea pig was taken as an indication of the actively progressing growth processes whereas the lesser amount in the scorbutic guinea pig was thought to be indicative of the lack of growth due to the deficiency state.

J. H. SHAW

The Effects of Ascorbic Acid Deficiency on the Teeth and Periodontal Tissues of Guinea Pigs. A. M. Hunt and K. J. Paynter. *J. Dent. Res.*, 38: 232, 1959.

Vitamin C deficiency was produced in guinea pigs under carefully controlled conditions of a reproducible semisynthetic diet of known composition, supplements of crystalline L-ascorbic acid and pair-feeding in an attempt to resolve some of the controversial issues in scurvy. The guinea pigs in group I were fed the basal diet *ad libitum* without supplementation for twenty-four days. The guinea pigs in the three subgroups of group II were fed for forty-eight days, respectively: the basal diet *ad libitum* with a daily supplement of 0.4 mg. of ascorbic acid per animal, the basal diet pair-fed to the intake of the deficient pigs supplemented by 5 mg. of ascorbic acid per animal per day, and the basal diet *ad libitum* with a 5 mg. supplement. The three subgroups in group III were fed identically except that the experimental period was extended to seventy-five days. The pigs in group IV were provided the basal diet with a supplement of 0.4 mg. per day for forty-eight days followed by a supplement of 5 mg. per day for an additional twenty-seven days.

The guinea pigs fed 5 mg. of ascorbic acid per day plus the basal diet *ad libitum* or pair-fed had no visible gross or microscopic abnormalities. When only 0.4 mg. of ascorbic acid was provided per day, some alterations in the structure of dentin were observed. Mild alteration

in the normal deposition and resorption of bone in the jaws began to appear between forty-eight and seventy-five days of deficiency. When the completely deficient diet was fed, more severe alterations were observed, with areas of hemorrhage in the pulp and in the periodontal membrane, a failure of connective tissue formation of collagen fibers, cementum, dentin and bone, and an excessive resorption of bone especially where areas of pressure normally occur. Enamel matrix failed to form in areas where predentin did not form. Cells of the stratum intermedium of the enamel organ proliferated at a level far apical to that at which proliferation ordinarily occurs and created the illusion that cells of the oral epithelium had migrated down the side of the tooth.

J. H. SHAW

The effect of preparation of foods upon their vitamin C content is an extremely important matter for consideration by dietitians and nutritionists.

Vitamin C Content of Cooked Vegetables. H Gounelle and M. Leclero. *Nutrition et Dieta (Basel)*, 1: 121, 1959.

Determination of vitamin C content of vegetables in dishes cooked under the conditions of a hospital kitchen has brought to light the following facts: (1) The residual concentration is low and very far removed from the content indicated in tables based on raw vegetables. Often there are mere traces only, in which case vitamin C intake is negligible. (2) The content is virtually the same whether the vegetables are fresh or canned. (3) Under the conditions of this study, cooked green and dried vegetables are equivalent: the amount of vitamin C finally supplied by either form, in terms of weight, is very small.

From these facts it is possible to predict vitamin C deficiency in hospital diets if the menu does not supply enough raw foods. But they may also point to the conclusion that the needs of the organism are less than commonly believed.

H. GOUNELLE

AMINO ACIDS AND NUTRITION

In considering the biologic value of protein, it is the combination of amino acids and the digestibility of the specific protein which determines its usefulness as a nutritive source. The assessment of the biologic value depends upon the percentage of digestible protein utilized by tissues and the provision of an appropriate "aminogram" for synthesis of new protein. Chemical scoring of protein dietary sources has been employed as one means of evaluation of foods.

Efficacy of the FAO Amino Acid Reference Standard for Growth of the Weanling Rat. E. E. Howe, E. W. Gilfillan and J. B. Allison. *J. Nutrition*, 70: 385, 1960.

The purpose of this study was to test out the reference amino acid pattern formulated by FAO for estimating the nutritional value of food proteins. Various mixtures of amino acids, casein and cottonseed flour were used in the experimental diets. All diets were isonitrogenous containing 1.8 per cent nitrogen, a level at which small critical changes in essential amino acid content are reflected in observed growth rates. Weanling rats were used in the study, which made it necessary to include arginine and histidine, amino acids not provided in the FAO reference pattern.

Decreasing the tryptophan content of the FAO pattern by 33 to 50 per cent or increasing the lysine content by 22 per cent did not alter the nutritive value of the mixture. A mixture containing casein supplemented to the FAO reference pattern with essential amino acids was found to be nutritionally equivalent to the FAO reference amino acid mixture itself, when fed at the same level of essential amino acid content.

The results suggest that casein is digested in such a way that the pattern absorbed is apparently the same whether the acids are fed free or combined in part as casein. Cottonseed meal supplemented with amino acids to the same FAO pattern proved to be inferior to the casein-supplemented mixture or to the reference mixture itself.

FRANK E. RICE

Supplementation of Cereal Proteins with Amino Acids. III. Effect of Amino Acid Supplementation of Wheat Flour as Measured by Nitrogen Retention of Young Children. R. Bressani, D. L. Wilson, M. Behar and N. S. Scrimshaw. *J. Nutrition*, 70: 176, 1960.

Diets containing wheat flour, gluten and glycine, with and without supplementation with essential amino acids, were fed to hospitalized children recovering from severe malnutrition; nitrogen retention was determined. Comparison of the amino acid content of the basic wheat protein diet with the FAO "reference protein" indicated an inadequacy in isoleucine, lysine, methionine, threonine, tryptophan and valine.

Lysine was demonstrated to be by far the most limiting amino acid. Supplementation with lysine alone was often sufficient to produce sustained nitrogen retention similar to that obtained from milk feeding, or, from the wheat diet supplemented according to the pattern of the FAO "reference protein." More consistent results, however, were obtained by tryptophan supplementation in addition to lysine, and also by the addition of the other limiting amino acids.

Methionine supplementation to a level as high as that indicated by the FAO proposal was not found necessary. It was concluded that the FAO "reference protein" is useful for determining the need for supplementing food proteins with amino acids.

FRANK E. RICE

The intracellular content of amino acids is increased by the action of insulin presumably by a change in cell membrane permeability affected by the hormone. A similar effect upon amino acid transport may be achieved with growth hormone.

Stimulation of Amino Acid Transport in Isolated Diaphragm by Growth Hormone Added in Vitro. J. L. Kostyo, J. Hotchkiss and E. Knobil. *Science*, 130: 1653 1959.

The isolated rat diaphragm was used to study the influence of pituitary growth hormone on the transport of amino acids into cells. In this experiment the non-utilizable amino acid, α-aminoisobutyric acid, was used. Measurements of the amount of amino acid nitrogen released into the medium indicated that the intact diaphragm preparation of a normal rat released somewhat more nitrogen into the medium than did that of a hypophysectomized animal. The addition of growth hormone had no effect on the rate of release of amino acid nitrogen.

It was found that the labeled amino acid enters the muscle cells of hypophysectomized rats at a lower than normal rate. Adding either simian or bovine growth hormone greatly enhanced the uptake.

The data support the hypothesis that pituitary growth hormone plays a role in the regulation of amino acid transport into muscle cells. Should growth hormone be found to stimulate transport of natural amino acids as well, then the effect of growth hormone may be through the initial increase in availability of amino acids. S. O. WAIFE

Imbalances in amino acid availability results in impaired protein synthesis with growth failure and a reduction in tissue nitrogen. These effects are widespread, and are evident in muscle, pancreas, liver and other glandular tissues. The organs affected will depend upon the supplies of the amino acids for which they have a high priority.

Chemical Pathology of Acute Amino Acid Deficiencies. III. Morphologic and Biochemical Changes in Young Rats Fed Valine- or Lysine-Devoid Diets. H. Sidransky and T. Baba. *J. Nutrition*, 70: 463, 1960.

Many of the pathologic lesions observed in rats ingesting diets deficient in certain amino acids resemble lesions described in the human disease, kwashiorkor. This is a third report of a series wherein rats are fed on amino acid-deficient diets. Force feeding and *ad libitum* feeding were employed; in each case the amino acid-deficient diets were compared with adequately balanced control diets.

The results obtained on force feeding were more significant. When the amino acid-deficient diets were offered *ad libitum*, the animals consumed much less; in consequence, deficiency symptoms were less marked at the end of the six day feeding period. Diets devoid of valine brought about the development of periportal fatty liver, excess of hepatic glycogen and atrophy of the pancreas, parotid, thymus and spleen. Lysine-devoid diets induced similar but less striking changes. Both diets when force-fed resulted in losses in body weight and increases in liver weight. With the same diets, however, provided *ad libitum*, there were decreases in the liver weights. "The pathologic lesions found in the rats force-fed valine- or lysine-devoid diets resembled those described to be characteristic of kwashiorkor." FRANK E. RICE

Histopathology of Amino Acid Deficiencies. VI. Effect of Arginine Deficiency in the Liver, with Consideration of the Testes and Accessory Sex Glands. E. B. Scott. *Arch. Path.*, 69: 390, 1960.

The following experiments were carried out in male rats of the Sprague-Dawley strain in order to determine whether or not arginine is essential for normal liver function: A diet free of arginine was fed to thirty or one-hundred day old rats for a period of seventy days. Since proline is convertible into arginine, twenty day old rats were fed an arginine- and/or proline-free diet for twenty-one days. The control animals were fed a diet containing nineteen crystalline amino acids. Some animals were pair-fed, others were fed the deficient diets *ad libitum*.

Despite the deficiency, the animals gained weight, and underfeeding was therefore ruled out as the cause of liver changes found at necropsy. In both immature and young adult animals, total omission of dietary arginine led to periportal fat infiltration. The changes became accentuated with increasing duration of the deficiency. Proline deficiency alone did not cause liver damage. The findings were similar to those produced by other amino acid deficiencies and seem to be of particular interest since arginine was not considered essential for the maintenance of normal liver function. In contrast to the results of studies by others, no changes were noted in the testes or accessory sex glands of the rats fed the arginine-free diet. M. SILBERBERG

Studies on the Effect of Dietary Protein and Caloric Levels on the Kinetics of Nitrogen Metabolism Using N^{15} L-Aspartic Acid. D. S. Tschudy, H. Bacchus, S. Weissman, D. M. Watkin, M. Eubanks and J. White. *J. Clin. Invest.*, 38: 892, 1959.

A fifty-three year old woman with lymphosarcoma was studied by an unusual technic which measures the metabolic pool and rate of protein synthesis under specialized conditions. Under the situation of a metabolic balanced study, the effect of variation in calories and protein content was determined. The rate constant for nitrogenous metabolic pool turnover did not appear to vary significantly with dietary changes. An increase in protein intake appeared to increase somewhat the size of the nitrogenous metabolic pool. The calculated rate of incorporation of nitrogen into protein increased when both calories and proteins were increased, even though the patient remained in nitrogen balance. The authors raise the question as to whether increased protein turnover is a prelude to positive nitrogen balance. This and related subjects are discussed in the article. S. O. WAIFE

NUTRITIONAL ASPECTS OF VITAMIN A

Both vitamin A and carotene are absorbed from the small intestine and are stored primarily in the liver. The ability to convert carotene to vitamin A may be traced to the presence or absence of specific proteins that can form complexes with carotene to (1) permit its absorption and (2) to facilitate its transport in blood and its conversion to vitamin A. Impairment of hepatic function induced by protein deficiency may affect the utilization of the vitamin directly.

Studies on the Utilization of Vitamin A. Part IV. Influence of the Level of Protein on the Storage and Utilization of Vitamin A. G. C. Esh, S. Bhattacharya and J. M. Som. *Ann. Biochem. & Exper. Med.*, 20: 15, 1960.

There is increasing evidence that protein metabolism is intimately related to the metabolism of vitamin A. It seems that protein may play a role in the proper absorption, transport, storage and utilization of vitamin A. In this study, the influence of three levels of protein (6, 12 and 18 per cent) in the diet on the storage and utilization of vitamin A at two levels (50 and 710 I.U. per day) has been studied in adult rats. Variations in the amount of protein, vitamin A and state of depletion or repletion were interrelated so that the authors concluded the level of protein did indeed have considerable effect on the utilization of vitamin A. It was also suggested that increased vitamin A storage in the liver, as observed with low protein intake, could not be utilized adequately. S. O. WAIFE

Reduction of cholesterol ester levels in patients with hypercholesterolemia by administering large doses of vitamin A is an interesting observation. If the depression of esterification is a toxic effect, it is difficult to understand the lack of reduction in normal patients.

Influence of Vitamin A on Cholesterol Blood Levels. L. J. Kinley and R. F. Krause. *Proc. Soc. Exper. Biol. & Med.*, 102: 353, 1959.

The oral administration of vitamin A, 100,000 units daily for four to six months, was associated with a fall in serum cholesterol levels of 20 to 175 mg. per 100 ml. in a group of hypercholesterolemic patients (initial mean serum cholesterol level 355 mg. per 100 ml.) who had sustained cardiac infarcts. The same dose, however, had no effect on the serum cholesterol levels of a group of healthy subjects aged thirty to fifty years. The effect in the hypercholesterolemic patients was confined to esterified cholesterol, the free fraction being unchanged. Cholesterol levels were determined by the Schoenheimer-Sperry method which is not affected by high vitamin A concentrations. Previous workers, using methods now known to be influenced by vitamin A, had claimed that the vitamin increased serum cholesterol levels. G. WALKER

Vitamin A deficiency following gastrectomy is an imtant observation which may be related to disturbances in protein metabolism commonly seen after this operation. The relation of vitamin A to protein intake has already been discussed.

Vitamin A Deficiency Following Total Gastrectomy. J. F. Adams, J. M. Johnstone and R. D. Hunter. *Lancet*, 1: 415, 1960.

Five cases of vitamin A deficiency in patients who had undergone total gastrectomy are reported. Serum vitamin A levels were between 3 and 10 μg./100 ml. Four subjects suffered from night blindness, the fifth had hyperkeratosis; all recovered when given vitamin A orally.

There was no apparent dietary deficiency of vitamin A and the origin of the deficiency may be complex. Steatorrhoea is not the only factor. It is suggested that vitamin A should be given prophylactically to all patients after gastrectomy. F. E. HYTTEN

The Effect of Vitamin A deficiency Upon Experimental Oral Carcinogenesis. N. H. Rowe and R. J. Gorlin. *J. Dent. Res.*, 38: 72, 1959.

A state of nutritional deficiency was produced in hamsters by feeding them a diet, prepared in the form of bread, composed of flour, lard, sugar, salt and yeast when they were forty-five days old and continuing for twenty weeks. The first group of hamsters received only the deficient diet, the second group received a weekly supplement of 400 I.U. of vitamin A, and the third group also received the vitamin A supplement but were pair-fed the amount consumed by the deficient hamsters. All hamsters in these three groups received an application of a 0.5 per cent solution of 7,12-dimethyl-1,2-benzanthracene in liquid petrolatum to the mocosa of the left buccal cheek pouch twice weekly for the first thirteen weeks of the experiment. The hamsters in groups four and five were identical with those in groups one and two except that the cheek pouch was swabbed with liquid petrolatum without the carcinogen.

After ingesting the deficient diet for five weeks, symptoms of mild vitamin A deficiency began to appear in the unsupplemented groups: the fur lost its characteristic smoothness and became slightly ruffled in appearance. This change in fur became marked by seven to eight weeks. Very slight xerophthalmia was evident by nine weeks and weight losses began after eleven weeks. The hamsters receiving the deficient diet lost an average of 4.5 gm. while those receiving the vitamin A supplemented diet *ad libitum* gained an average of 6.3 gm. The hamsters receiving the supplemented diet restricted in food intake lost an average of 4 gm. The poor growth in the control animals ingesting the supplemented diet suggests that the diet was deficient in more than vitamin A. The serum of the animals eating the deficient diet contained 19 μg. per cent of vitamin A while that of the hamsters receiving the supplemented

diet varied from 57 μg. per cent five hours after supplementation to 26 μg. per cent after a week.

The incidence of clinically observable tumors was greater in the group receiving the unsupplemented diet than in either of groups two and three receiving the supplemented diet. No lesions were observed in groups four and five.

On microscopic examination, the mucosa of the pouch was graded into six categories: (1) normal, (2) vitamin A deficient, (3) hyperkeratotic, (4) benign papilloma, (5) carcinoma *in situ*, and (6) invasive squamous cell carcinoma. Tumors were observed in 81.5 per cent of the twenty-seven deficient hamsters and malignancies in 59 per cent. Among the twenty-seven hamsters receiving the vitamin A supplemented diet 74 per cent had tumors and 33 per cent had malignancies. Among the twelve hamsters receiving the vitamin A supplemented diet and pair-fed, 58.3 per cent had tumors and 25 per cent had malignancies.

These data suggest that vitamin A deficiency increased the susceptibility of hamsters to carcinogen-induced benign and malignant tumors. It is unfortunate that these studies were not conducted with a diet that was more clearly deficient in vitamin A without the suspicion of other simultaneous deficiencies.

J. H. SHAW

Hypervitaminosis A has become a recognized clinical syndrome in children and adults. The tendency to include a high content of the vitamin in many of the commercially available supplements taken without supervision may be unfortunate.

Oxidative Processes in Hypervitaminosis A in Albino Rats. A. Ray and D. P. Sadhu. *Am. J. Physiol.*, 196: 1274, 1959.

Hypervitaminosis A was induced in albino rats by feeding them 30,000 I.U. of vitamin A daily orally; the effect of this hypervitaminosis was compared with a similar pair-fed group. Food consumption and body weight gain were reduced.

Study of liver and kidney slices shows that the latter manifest no significant increase in oxygen consumption in the presence of succinate or acetate. There is slight increase in O_2 consumption in brain homogenates. Liver homogenate shows 15.2 per cent inhibition with succinate and 5.6 per cent with ascorbate oxidation. Liver homogenate shows 19.3 per cent inhibition of succinate dehydrogenase activity by Thunberg technic with methylene blue indicator.

It is concluded that hypervitaminosis A inhibits liver respiration by affecting the dehydrogenase, or any immediate step following it, and the cytochrome *c*-cytochrome oxidase end of the succinoxidase system is little affected. AUTHORS

Study of Intestinal Absorption by Providing Experimental Hypervitaminosis. A Vachon and P. Dissard. *Semaine hôp Paris*, 51: 2975, 1959.

Intestinal absorption can be studied by experimental hypervitaminemia provoked by ingestion of 500,000 units of vitamin A. This was accomplished in patients with medical diseases of the small intestine (steatorrhea, intestinal scleroderma) and in surgical patients who had undergone extensive ileal resection or ileosigmoid anastomosis, as well as in control subjects. The level remained low in all cases and the greatest differences in the level were less than 100,000 I.U. The findings were the same in three patients after gastrectomy who had intestinal disorders and a deficiency syndrome.

Analysis of an Anomalous Oculodentofacial Pattern in Newborn Rats Produced by Maternal Hypervitaminosis A. F. M. Deuschle, J. F. Geiger and J. Warkany. *J. Dent. Res.*, 38: 149, 1959.

A detailed study has been conducted on the influence of toxic single doses of 75,000 to 150,000 I.U. of vitamin A given to pregnant rats. Gross developmental malformations of the fetuses occurred with a striking exophthalmia with open eyes and shortening of both the mandibles and maxillae resulting in protrusion of the tongue. The eyes, although large and lidless, were otherwise almost normal in structure but apparently were caused to protrude because of skeletal anomalies. These anomalies included a posterior cleft palate, an ankylosis due to a narrow bony bridge between the lateral part of the maxilla and the mandible, a well developed mass of heterotopic maxillary cartilage between the maxilla and the mandible, and varying degrees of deficiency in the alveolar processes and molar teeth. As many as eleven of the twelve molars were missing in some of the eight specimens that were sectioned serially. In other animals the molars that were present were hypolastic. Thus, the syndrome manifested itself chiefly in derivatives of the mandibular arch and it appeared likely that the eyes were secondarily involved because of their regional relationship to this area. Mesodermal and ectodermal structures were affected and it was postulated that the dental anomalies were attributable to the anomalies of the underlying mesodermal tissues.

This hypervitaminosis syndrome was different from that of riboflavin deficiency in several respects. In the fetuses influenced by riboflavin deficiency, only incisor teeth were affected with the lower more than the upper, and the distal portions of the jaws were reduced in length instead of the proximal portions. J. H. SHAW

ITEMS OF GENERAL INTEREST

The Binding between Electrolytes and Serum Proteins Calculated from an In Vivo Filtration Method. J. Gerbrandy, A. M. van Leewen, H. B. A. Hellendoorn and L. A. De Vries, with J. J. van Daatselaar. *Clin. Sc.*, 19: 181, 1960.

If a sphygomanometer cuff is inflated around the upper part of the arm to just below the systolic blood pressure, venous pressure builds up quickly (30 seconds

if the fist is clenched) to the pressure in the cuff. This raises the pressure in the capillaries and so increases filtration across their walls.

This method has been used in an ingenious experiment to show the proportion of various blood constituents bound to the plasma protein and therefore nonfilterable. The changes are reported in detail and cannot be adequately condensed here, but, as an example, plasma protein content rose by an average of 20 per cent, without changes in its relative proportions, after seven minutes at a pressure of 90 mm. Urea and glucose appeared to be completely filterable whereas alkaline phosphatase, total cholesterol, total bilirubin and iron were completely nonfilterable. Of the cations, the following proportions appeared to be nonfilterable, Na 8 to 10 per cent, Ca 34 to 43 per cent and Mg 45 per cent. Some phosphate also appears to be protein bound. Many of these findings will not be new to biochemists, but when so much research is presently conducted on the basis of blood composition this paper emphasizes how careful the investigator must be in the collection of his samples. F. E. HYTTEN

Food Composition Tables in the U.S.A. E. Neige Todhunter. *J. Am. Diet. A.*, 37: 209, 1960.

Food composition tables have become highly detailed since "Bulletin 28," *The Chemical Composition of American Food Materials*, authored by W. O. Atwater and C. D. Woods, was published by the U. S. Department of Agriculture in 1896. This bulletin, which gives data on the minimum, maximum and average percentage of refuse, water, protein, fat, carbohydrate and the fuel value per pound of a variety of American food products, contains data from 2,572 analyses. Dr. Todhunter tells in a dynamic fashion the evolution of U. S. food composition tables from 1896 to 1959 when the most recent publication, *Fatty Acids in Food Fats*, was published by the institute of Home Economics, USDA.

Food composition data have been presented as well in terms of quantities of food commonly eaten (Bowes and Church, *Food Values of Portions Commonly Served*). Compilations of this type furnish a quickly assessed, practical set of food values for students of medicine, dentistry, dental hygiene and public health nursing. They have been widely used by nutritionists.

The listing of government and nongovernmental publications and technical as well as practical tables of food value along with several "short methods" of dietary assay makes this an invaluable article for persons wishing to know the story of food composition.

J. M. SMITH

The Span from Rat to Man. R. H. Barnes. *New York J. Med.*, 59: 2931, 1959.

The problem of extrapolating from animals to man is discussed briefly. Species differences are well known: examples are the relative absence of an ascorbic acid requirement by certain species and the inability of a vitamin D-free diet to produce rickets in rats if the ratio of phosphorus to calcium is high. However, as the experience with pyridoxine in man indicates, merely being unable to demonstrate a nutritional requirement in man has led to the assumption that man differs from the experimental animal in nutritional requirements. Similarly animal experiments thirty years ago showed the essentiality of certain fatty acids. Only now is even the skeptic convinced that linoleic acid, for example, is essential for man.

The problem of species differences is best seen with reference to clinical and experimental atherosclerosis. The well known lack of correlation in findings emphasizes the need to establish firmly the fundamental cellular or subcellular biochemical metabolic changes involved. S. O. WAIFE

Complement-Fixing Autoantibody to Thyroglobulin in Hashimoto's Disease. J. R. Anderson, R. B. Goudie and K. G. Gray. *Lancet*, 2: 644, 1959.

There has been strong evidence, since 1956, that precipitating antibodies can be demonstrated in the serum of patients with Hashimoto's disease and some patients with other thyroid abnormalities. The antigen has been human thyroglobulin.

The present authors performed complement fixation tests on the serum of patients with Hashimoto's disease, using saline extracts of normal human thyroid tissue, as well as saline extracts of colloid-goiters, as test antigens. Positive reactions were observed with nine of fifty-three serum samples tested. Of the positive serum specimens, four were investigated further. It was found that colloid-goiter extracts had a higher titer (1:8,000), than thyroid extracts (1:2,000). A relationship was postulated of the amount of colloid present in a gland and its antigenic titer in complement fixation reactions. This hypothesis was made because lower titers were found with antigenic extracts of glands that contained small amounts than those glands with greater amounts of colloid. The antigen again was believed to be thyroglobulin.

On the basis of their work the authors' have described another type of antibody to thyroglobulin, one which fixes complement. Other workers have previously reported precipitins and antibodies that agglutinate thyroglobulin-coated tanned red cells. The real question of which antibody is responsible for the destructive process in the gland has not been answered. It is easy to be overwhelmed by the complexities of the immunological technics used in these investigations. At any rate, as stated by the authors, it is uncertain that the antibodies described are responsible for the pathogenesis of Hashimoto's disease. E. COHEN

A Screening Test for Malabsorption Syndromes. A. W. Horsley, J. A. Clifton, W. E. Connor and T. C. Evans. *Proc. Soc. Exper. Biol. & Med.*, 100: 588, 1959.

Plasma turbidity after a fat-containing meal depends partly on the rate of absorption of fat from the gut. Assessment of fat tolerance by repeated measurements

of plasma optical density after taking a standard fat meal containing 50 gm. corn oil is inexpensive and gives an estimation of fat absorption which compares favorably with more tedious and expensive methods, including studies of fat balance, serum carotene and I^{131}-labeled fat absorption.

Patients with malabsorption syndromes show a subnormal rise of plasma optical density after the test meal. In some instances improvement of their clinical condition is accompanied by increased postabsorptive plasma optical density values. G. WALKER

Carbohydrate Tolerance of Tube-Fed Rats, Main Effects and Interactions of Insulin, Growth Hormone and Carbutamide. J. A. Owen, Jr. *Diabetes*, 8:51, 1959.

In these very cleverly designed experiments adult rats, tube-fed with a high carbohydrate diet, were given insulin, growth hormone and carbutamide singly, and in all possible combinations, for four days. The effects of these drugs and their various combinations on blood glucose, nitrogen balance and weight gain, were analyzed for major effects and interactions by the analysis of variance applied to a 2^3 factorial design. Carbutamide produced highly significant hypoglycemia, while that produced by insulin was barely significant. Growth hormone effected significant weight gain and nitrogen retention as well as hyperglycemia. No other significant effects were produced by any of the drugs or combinations thereof. Carbutamide appeared to stimulate insulin release via mechanisms which do not respond to the hyperglycemia produced by growth hormone.

G. HOLLIFIELD

Antiinflammatory Activity of Some Water-Soluble Bioflavonoids. V. Menkin. *Am. J. Physiol.*, 196: 1205, 1959.

The water-soluble citrus bioflavonoid mixture contained in the preparation C.V.P., without added vitamin C, is capable of inhibiting the increased capillary permeability referable in turn to an inflammatory exudate, irrespective of the pH of that exudate. The suppressing effect of these water-soluble bioflavonoids can be demonstrated in concentrations of 5 or 10 mg./0.25 ml. of exudative material. They likewise inhibit the effect of leukotaxine that has been extracted from alkaline exudates. In this way they repress not only increased permeability of capillaries, but also the local emigration of leukocytes. Finally this water-soluble bioflavonoid mixture appears to behave as an antiinflammatory agent over a larger pH range of exudates than either cortisone or corticotropin (ACTH). Cortisone represses permeability increase and leukocytic migration of alkaline exudate. ACTH suppresses the activity of exudin in its ability to increase local capillary permeability induced by acid exudates. This bioflavonoid mixture is capable of inhibiting in this regard both alkaline and acid exudates. The possible potentials of C.V.P. are pointed out. AUTHOR

Transfer of Vitamins from Mother to Fetus. B. F. Chow and K. Okuda. *J.A.M.A.*, 172: 422, 1960.

This paper reviews some previously published work by these investigators. The concentrations of vitamin B_{12} in umbilical cord blood compared with concentrations in corresponding samples of maternal serum at the time of delivery indicate that the fetal concentrations are higher than the maternal. This is interpreted to be the result of active transfer of vitamin B_{12} from mother to fetus. In addition, iron, pyridoxine and ascorbic acid were present in higher concentration in fetal than in maternal blood. However, this was not true for glucose and inulin. S. O. WAIFE

Physiological Disposition of D- and L-Thyroxine in the Rat. D. F. Tapley, F. F. Davidoff, W. B. Hatfield and J. E. Ross. *Am. J. Physiol.*, 197: 1021, 1959.

A comparison was made of the distribution and excretion of the optical isomers of thyroxine in the rat. D-Thyroxine disappeared more rapidly from the blood and was concentrated to a greater extent in the liver and kidney than was L-thyroxine. The concentration of D-thyroxine in such peripheral tissues as muscle, brain and skin was much less than the concentration of L-thyroxine. This difference in distribution to the peripheral tissues was of such magnitude as to suggest a possible basis for the different activities of the two isomers observed *in vivo*. The half-time of D-thyroxine in the body (fourteen hours) was significantly shorter than that of L-thyroxine (forty-two hours). AUTHORS

Trypsin, Invertase and Amylase Content of Feces of Germfree Rats. R. Borgström, A. Dahlqvist, B. E. Gustafsson, G. Lundh and J. Malmquist. *Proc. Soc. Exper. Biol. & Med.*, 102: 154, 1959.

Trypsin and invertase were found in the feces of germfree rats but not of ordinary laboratory rats. The enzymes were not destroyed by infecting the germfree animals with spores from the feces of ordinary rats, but disappeared after feeding small amounts of unheated feces. These results suggest that trypsin and invertase are inactivated in the rat gut by bacterial action rather than by digestive enzymes secreted into the gut. Amylase was found in the feces of both germfree and ordinary animals and appears to resist destruction by both digestive and bacterial enzymes.

G. WALKER

The Concept of Egg Yolk as a Dietary Inhibitor to Rheumatic Susceptibility. A. F. Coburn. *Lancet*, 1: 867, 1960.

Although recent advances have established the streptococcus as the infective factor in rheumatism, there is still very little known about susceptibility to the disease. In this paper the evidence is assembled and discussed for the view that a sufficiency of egg yolk in the diet is an important influence in reducing susceptibility. Some of the more circumstantial evidence is that "rheumatic families," known generally to be poorer than average, have a low egg consumption and that within the family nonrheumatic siblings eat significantly

more eggs than those with rheumatism. Improvement in the diet reduced recurrences: this was true when the diet was supplemented either with egg yolk or an alcohol soluble fraction of it.

N-(2-hydroxyethyl)-palmitamide has been identified in animals as an antiallergic component of this fraction and is being tested as a dietary supplement among poor children in New York City. There is no information about any possible biochemical mechanism which could explain the suggested relationship. F. E. HYTTEN

Brain Lesions in Chronic Alcoholism. M. J. G. Lynch. *Arch. Path.*, 69: 342, 1960.

The changes taking place in the brains of eleven habitual heavy drinkers of long standing were studied. The livers of all these patients, who were in "a good state of nutrition" at the time of death, showed fatty metamorphosis and eight were cirrhotic. Grossly, the brains showed moderate cortical atrophy with dilatation of the ventricles, thickening of the leptomeninges and minute porosities in the white matter about the internal capsule. Microscopically, there was loss of 20 to 50 per cent of the pyramidal cells, of the Purkinje cells, and of cells of the basal and dentate nuclei as well as of the anterior horns. Furthermore, there were throughout the brain increased vascularity, widening of the Robin-Virchow spaces, minute perivascular hemorrhages with aggregations of lipid-laden mononuclears, foci of demyelination and loss of nerve fibers. Additional findings were fat emboli in the small arteries of the brain, the choroid plexus and also of the lungs. It is thought that the brain lesions are the result of the response to repeated fat emboli which occurred at various times during life. M. SILBERBERG

Tables of Average Weight and Height of Americans Aged 65 to 94 Years. A. M. Master, R. P. Lasser and G. Beckman. *J.A.M.A.*, 172: 658, 1960.

Standards of height and weight were constructed from data obtained from subjects in all geographic areas of the United States, belonging to all social and economic levels, of various ethnic origins and engaged in a variety of occupations. All were ambulant and apparently in good health. These subjects had no evidence of cardiovascular or renal disease. There were 2,925 men and 2,694 women in the group. Heights and weights were measured in stocking feet, and weight included that of light undergarments.

In both sexes and at all heights, the average weight became lighter with advancing age. Height, unlike weight, virtually did not diminish with further aging. These valuable figures are averages of actual measurements rather than optimal or ideal figures. This is the first table to be so constructed for this geriatric group. S. O. WAIFE

The Effect of Diet on Growth Rate and Feed Efficiency in the Normal Rat. C. H. Lushbough and B. S. Schweigert. *J. Nutrition*, 70: 252, 1960.

Dietary factors particularly studied were level of fat, kind of fat (lard or corn oil) and level of protein. Casein was the protein employed in all diets. Sucrose, the carbohydrate used, ranged from 10.7 to 83 per cent depending on the caloric density desired. Male weanling rats, twenty-five days old, were employed. Water and food were provided *ad libitum* during the eight-week experimental period.

Progressive increases in protein: calorie ratios over a broad range (11.8 to 47.2 per cent) produced consistent increases in weight and in efficiency of feed utilization when either lard or corn oil provided 11.1 or 44.2 per cent of the calories in the experimental ratios. When the fat: calorie ratio was increased, there resulted increased weight gains and feed utilizations if the protein intakes were adequate. When protein was at growth-limiting levels, however, dilution of the ration by either fat or sucrose brought about a reduction of biologic performance.

No significant differences in response to the feeding of lard or corn oil were observed. FRANK E. RICE

A New Treatment of Anorexia Nervosa. P. J. Dally and W. Sargant. *Brit. M. J.*, 1: 1770, 1960.

Despite many vogues there has never been a satisfactory treatment for the dangerous condition of anorexia nervosa. The method described here offers the best results published so far and will be a relief to any physician faced with this problem.

The patients are kept in bed until normal weight is regained. Chlorpromazine is given orally starting with 150 mg. daily and increasing by 75 mg./day until the limit of tolerance. Insulin therapy is also started at once beginning with 5 units and increasing this until the patient sweats and becomes drowsy. Beginning with a light diet of 1,500 calories, food intake is increased to about 4,000 calories daily. Psychotherapy may follow the physical recovery: often chloropromazine therapy is continued.

Twenty patients, nineteen of them women, treated in this way gained 4.4 ± 1.3 pounds of weight weekly and the average stay in hospital was 34.9 ± 12.2 days. Three required readmission once and one, twice. F. E. HYTTEN

Results of a Study of 100 Cases of Gout. S. de Sèze, A. Ryckewaert, J. Levernieux and R. Marteau. *Semaine hôp. Paris*, 34: 2289, 1959.

From study of 100 authentic cases of gout, the authors conclude that the aspect of gout has hardly changed since the days of Sydenham. They consider it useful to recall certain classic signs: the hereditary aspect, frequency in males, the effect of overnutrition and alcoholism, the occurrence and development of episodes, the tophi, urate arthropathy and lithiasis. They discuss treatment and its indications (colchicine, phenylbutazone, ACTH, delta-hydrocortisone, probenecid and salicylates). H. GOUNELLE

Biological Determination of Gluco- and Mineralocorticoid Substances in the Lung. I. G. Fàzekas. *Ztschr. Vitamin-Hormon- u. Fermentforsch.*, 11: 29, 1960.

Using the method elaborated by Swingle and Pfiffner for the preparation of adrenalcortical extracts, an extract was prepared from the lungs of freshly slaughtered pigs. This extract contained no protein, fat or cholesterol, no fatty acids and no bile pigment. The active principle of the extract was readily soluble in 70 to 96 per cent ethanol, in benzene, acetone and water, and displayed a potent reducing effect.

In adrenalectomized male mice, this pulmonary extract increased the glycogen content of the liver and musculature, its effect in this respect being dependent upon the size of the dose administered. In intact male rats it likewise increased the glycogen content of the liver and musculature, and raised the blood sugar level. The glycogen content of the brain was only slightly enhanced by the extract in adrenalectomized mice and showed no change in intact rats. In rabbits, the extract increased the sodium content of the blood serum, while on the other hand it diminished the potassium content.

From these results, it may be concluded that the lungs contain substances which exert the same biologic effect as the glucocorticoids and mineralocorticoids of the adrenal cortex. The author is of the opinion that these substances are in fact identical with the corticosteroids and that the lungs are to some extent capable of storing corticosteroids. AUTHOR

The Agglutination and Sequestration of Immature Red Cells. J. H. Jandl. *J. Lab. & Clin. Med.*, 55: 663, 1960.

During studies of iron metabolism in reticulocytes from various species, it was noted that immature red cells possessed properties usually encountered in mature red cells coated with antibodies. Immature red cells of man, rabbit and rat were found to be selectively agglutinated by Coombs' serum and by polyvinylpyrrolidone (PVP). At least part of the protein coating of immature red cells was found to be due to a protein immunologically similar to transferrin. Specific agglutination of reticulocytes by Coombs' serum was found in six of ten patients who had a variety of disorders with reticulocytosis. Strong clumping of rabbit or rat reticulocytes were found with appropriate Coombs' serum. Splenic trapping of reticulocytes, rather than a splenic hormone, was suggested as the cause of suppressive effect of spleen on erythropoiesis in certain patients believed to be caused by their increased agglutinability and greater size as compared to mature cells. Are transferrin or other red cell coating proteins part of a mechanism for release of newly formed red cells from marrow sinusoids? The authors have not answered that question, but their report is a significant step in that direction. E. COHEN

All-Vegetable Protein Mixtures for Human Feeding. III. The Development of INCAP Vegetable Mixture 9. R. Bressani, L. G. Elias, A. Aguirre and N. S. Scrimshaw. *J. Nutrition*, 74: 201, 1961.

Reported here is the continuation of experiments to find a vegetable protein mixture satisfactory in nutritive value for use in underdeveloped areas of the world where milk and other animal products are costly or limited. Earlier experiments by these authors have demonstrated that sesame flour has high nutritive value. For Central America, however, cottonseed flour is a more practical vegetable protein source. In these experiments the substitution of the latter for the former gave satisfactory results with respect to growth and feed efficiency with rats and chicks. The use of corn and sorghum (lime-treated) in the experimental mixtures was investigated, as these products are also readily available in Central America. The proteins of these grains are deficient in the amino acids, lysine, tryptophan and isoleucine, but the higher values in cottonseed flour tend to complement these deficiencies. After experimentation with various combinations, the authors arrive at "a new formula for potential human consumption called INCAP Vegetable Mixture 9:" ground yellow corn, 28 per cent; ground sorghum grain, 28 per cent; cottonseed flour, 38 per cent; Torula yeast, 3 per cent; dehydrated kikuyu leaf meal, 3 per cent. In essential amino acid composition Mixture 9 is shown to compare quite favorably with FAO Provisional Reference Protein, methionine being the most limiting amino acid, followed by tryptophan, then lysine and isoleucine (acid hydrolysis values). Comparisons, using enzymatic digest values, however, shows lysine to be most limiting followed by methionine and tryptophan as compared with the FAO Reference Protein.

FRANK E. RICE

All Vegetable Protein Mixtures for Human Feeding. IV. Biological Testing of INCAP Vegetable Mixture 9 in Chicks. R. Bressani, A. Aguirre, L. G. Elias, R. Arroyave, R. Jarquin and N. S. Scrimshaw. *J. Nutrition*, 74: 209, 1961.

In this second paper possible substitutions in Formula 9 were investigated using the chick as the experimental animal. It was found that the proportions of corn and sorghum could be changed without altering the nutritive value of the mixture, also that rice can well be substituted, which may be desirable in those areas where it is the more readily available cereal. Brewers' yeast could not be substituted for Torula yeast; its bitter taste apparently lowers feed consumption. Other carotene-rich leaf meals could be substituted for kikuyu leaf meal, so also can synthetic vitamin A preparations, when good quality leaf meals are not available. For the chick the lysine in the mixture is only about 83 per cent mostly due to a lower availability of the lysine in cotton seed. Supplementation of the mixture with methionine did not improve growth or feed efficiency. "It was concluded that Vegetable Mixture 9 is of high

nutritional value and of potential value in human feeding." FRANK E. RICE

Changes in Plasma Free Fatty Acid Concentrations on Passage Through the Dog Kidney. F. J. Hohenleitner and J. J. Spitzer. *Am. J. Physiol.*, 200: 1095, 1961.

To measure the renal removal of free fatty acids from the plasma, simultaneous determinations of this metabolite were performed in a systemic artery and a renal vein in the anesthetized dogs. Renal plasma flow was also determined by the PAH method, and the renal uptake of free fatty acids was calculated. Concentrations of free fatty acids in renal venous plasma were usually lower than the arterial concentrations. The arteriovenous differences were statistically highly significant. The results also suggested that the degree of free fatty acid removal was proportional to the arterial concentration of this metabolite. AUTHORS

A Comparison of the Absorption and Retention by Rabbits of Strontium-90 Incorporated into Plant Material and of Added Strontium Chloride in the Diet. T. E. F. Carr and J. Wall. *Brit. J. Nutrition*, 15: 165, 1961.

Sr-90 fallout may reach man from edible plants that have absorbed the nuclide through their roots or from that deposited on the surface of the vegetation. In this study it is assumed that strontium chloride added to the diet should be similar in availability to surface contamination. For comparison, grass grown on soil to which Sr-90 had been added was fed as "incorporated strontium." Carcass analyses were made after short (twenty-four hours) and long (twenty-one days) feeding periods.

No difference was found in the behavior of these two forms of strontium after their administration to the rabbit. It was concluded that the pressence of phytates or other organic materials found in plants does not affect strontium absorption at the gut.

FRANK E. RICE

Active Transport of Calcium by Intestine: Action and Bio-Assay of Vitamin D. D. Schachter, D. V. Kimberg, and H. Schenker. *Am. J. Physiol.*, 200: 1263, 1961.

Vitamin D is required for the active transport of calcium *in vitro*. Small doses of vitamins D_2 and D_3 restore the mechanism in depleted rats, and this provides a sensitive bio-assay for the vitamin, independent of an antirachitic effect. Vitamin D influences calcium transfer in all segments of the small intestine, and maximal increments are observed in the duodenum. The effect of vitamin D requires oxidative metabolism *in vitro*, is maximal where active transport is maximal, and the sterol increases the maximal rates of active transport of calcium. Consequently, vitamin D influences calcium transport by affecting primarily the active mechanism rather than by simple diffusion. Experiments with various monosaccharides demonstrate that two distinct steps are involved in the active transport mechanism in duodenum. Vitamin D is required for both of the steps. AUTHORS

Diet and Serum Cholesterol of Lumberjacks. M. J. Karvonen, M. Pekkarinen, P. Metsälä and Y. Rautanen. *Brit. J. Nutrition*, 15: 157, 1961.

Serum cholesterol levels of lumberjacks in Finland were not significantly different from that of other males in the local population, although fat intakes were much greater. Food consumption of the lumberjacks averaged 4,763 calories per head per day, 45 per cent of which was derived from fat. By comparison, the local population averaged 2,732 calories per individual, 35 per cent of which came from fat. In grams of fat per day, lumberjacks consumed 237, local population 105. The ratios of saturated to polyenoic fatty acids were similar in both cases, 7.0 to 7.5.

Serum cholesterol levels were high in both groups, well over 260 mg. per 100 ml. in the upper ages, but about the same at comparative ages. The authors conclude that the higher intake of fat by lumberjacks with its supposed cholesterogenic effect was balanced by the heavier physical work. FRANK E. RICE

In Memoriam

NORMAN HAYHURST JOLLIFFE, B.S., M.D., F.A.C.P.

On August 1, 1961, clinical nutrition in America lost one of its most significant figures. Norman Jolliffe, Director, since 1949, of the Bureau of Nutrition of the Department of Health of the City of New York, died at the age of fifty-nine, after a protracted illness.

Dr. Jolliffe, a native of West Virginia, graduated from West Virginia University in 1923 and received his M.D. degree from New York University and Bellevue Hospital College of Medicine in 1926. After an internship and residency at Bellevue, and two years in the department of physiology at the medical school, he became chief of the medical service on the psychiatric division of Bellevue Hospital in 1932.

At that time the patient population of the psychiatric division included a large number of malnourished persons who exhibited a variety of organic and emotional disorders. Dr. Jolliffe early recognized the possibility of a cause and effect relationship and with great enthusiasm and competence both undertook to explore it himself and to encourage his associates to do so. His work here, particularly with chronic alcoholics, brought him quick recognition as a pioneer and international authority on clinical nutrition.

During World War II, he was a member of the Food and Nutrition Board of the National Research Council and was one of the original and most effective advocates of the enrichment of white bread and flour. At the request of various agencies and governments he participated in nutrition surveys and studies not only in this country, but also in Newfoundland, Formosa, Brazil, Cuba and Italy. In 1955 he received the Carlos J. Finley Award from the Cuban Government for his work in that country.

When Dr. Jolliffe, in 1949, accepted the position of Director of the Bureau of Nutrition, New York City Department of Health, he also accepted the responsibility to give the city its money's worth and a bureau of which it could be proud. In this he eminently succeeded. In addition to its neighborhood and consultative nutrition services, the Bureau now operates six general nutrition clinics, three obesity clinics and an "Anti-Coronary Club," all medically directed and staffed by physicians and nutritionists.

The Diet and Coronary Heart Disease Study Project (the "Anti-Coronary Club") was set up by Dr. Jolliffe in 1957 to see if practical, blood cholesterol-reducing diets acceptable to free-living men could be developed, and if such diets adhered to over a period of time would result in a decreased incidence of coronary accidents. This work was his major interest in the last years of his life, and no amount of physical incapacity prevented him from giving it his full attention. It is pleasant to be able to record that it is being continued as he would have wished.

Dr. Jolliffe was a distinguished physician and his affiliations and interests were many, but he never lost interest in clinical nutrition and was a never failing source of encouragement to younger physicians in this field. He was a charter member and one of the incorporators of The American Society for Clinical Nutrition. His contributions to the medical literature were many. The second edition of the textbook "Clinical Nutrition," first published in 1950 and revised under his editorship shortly before his death, is scheduled to appear in January 1962.

Dr. Jolliffe is survived by his wife, Lillian; a son, Norman, Jr.; two granddaughters; his mother; a brother and three sisters. He shall be missed by his many friends and associates.

R.S.G.

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AND

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